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(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF BCL2 GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

(57) Abstract: The present invention concerns methods and reagents useful in modulating BCL2 gene expression in a variety of applications, including use in therapeutic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), doublestranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against BCL2, BCL-XL, MCL-1, BCL2-Ll, CED-9, BAG-1, ElB-194 and/or BCL-A1 gene expression, useful in the treatment of cancer and any other condition that responds to modulation of BCL2, BCL-XL, MCL-1, BCL2-Ll, CED-9, BAG-1, E1B-194 and/or BCL-A1 expression.

RNA INTERFERENCE MEDIATED INHIBITION OF BCL2 GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

This invention claims the benefit of McSwiggen USSN 60/396,905 filed July 18, 2002, of Beigelman USSN 60/358,580 filed February 20, 2002, of Beigelman USSN 60/363,124 filed March 11, 2002, of Beigelman USSN 60/386,782 filed June 6, 2002, of Beigelman USSN 60/406,784 filed August 29,2002, of Beigelman USSN 60/408,378 filed September 5, 2002, of Beigelman USSN 60/409,293 filed September 9, 2002, and of Beigelman USSN 60/440,129 filed January 15, 2003. These applications are hereby incorporated by reference herein in their entireties, including the drawings.

Field Of The Invention

The present invention concerns compounds, compositions, and methods for the study, diagnosis, and treatment of conditions and diseases that respond to the modulation of BCL2 gene expression and/or activity. The present invention also concerns compounds, compositions, and methods relating to conditions and diseases that respond to the modulation of expression and/or activity of genes involved in BCL2 pathways. Specifically, the invention relates to small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against BCL2 gene expression.

Background Of The Invention

The following is a discussion of relevant art pertaining to RNAi. The discussion is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature,* 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of

foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877) has revealed certain requirements for siRNA

length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21-nucleotide siRNA duplexes are most active when containing 3'-terminal dinucleotide overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with 2'-deoxy nucleotides (2'-H) was shown to be tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end of the guide sequence (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309).

Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two -nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). In addition, Elbashir *et al.*, *supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.*, International PCT Publication No. WO 00/44914, and Beach *et al.*, International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA may include modifications to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer *et al.* similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in siRNA molecules.

Parrish *et al.*, 2000, *Molecular Cell*, 6, 1977-1087, tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases also had substantial decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides produced a substantial decrease in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil substitution appeared to be tolerated, Parrish reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila in vitro* RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of particular genes in mammalian cells using certain dsRNA molecules. Fire *et al.*, International PCT Publication No. WO 99/32619, describe particular methods for introducing certain dsRNA molecules into cells for use in inhibiting gene expression. Plaetinck *et al.*, International PCT

Publication No. WO 00/01846, describe certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific dsRNA molecules. Mello *et al.*, International PCT Publication No. WO 01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Deschamps Depaillette *et al.*, International PCT Publication No. WO 99/07409, describe specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.*, International PCT Publication No. 99/53050, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.*, International PCT Publication No. WO 01/49844, describe specific DNA constructs for use in facilitating gene silencing in targeted organisms.

Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.*, 2000, *Molecular Cell*, 6, 1977-1087, describe specific chemically-modified siRNA constructs targeting the unc-22 gene of *C. elegans*. Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.*, International PCT Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs. Cogoni *et al.*, International PCT Publication No. WO 01/53475, describe certain methods for isolating a Neurospora silencing gene and uses thereof. Reed *et al.*, International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.*, International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as Parkinson's Disease models using certain dsRNAs. Deak *et al.*, International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that may be related to RNAi .in *Drosophila*. Arndt *et al.*, International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.*, International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.*, International PCT Publication No. WO 00/63364, and Satishchandran *et al.*, International PCT Publication No. WO 01/04313, describe certain methods and compositions for inhibiting the function of certain polynucleotide sequences using certain dsRNAs. Echeverri *et al.*, International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.*,

International PCT Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using RNAi. Graham *et al.*, International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.*, US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain siRNA constructs that mediate RNAi.

Lin *et al.*, International PCT application No. WO 02/10374, describes a certain gene silencing approach using particular mRNA-cDNA duplexes targeting BCL2 expression.

Warrel *et al.*, International PCT Publication No. WO 02/17852, describes certain BCL2 antisense oligonucleotides.

Thompson *et al.*, US 5,750,390, describes nucleic acid mediated inhibition of BCL2 expression.

SUMMARY OF THE INVENTION

This invention relates to compounds, compositions, and methods useful for modulating BCL2 expression by RNA interference (RNAi) using small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules. This invention also relates to compounds, compositions, and methods useful for modulating the expression and activity of BCL2 genes, or genes involved in BCL2 pathways of gene expression and/or BCL2 activity by RNA interference (RNAi) using small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules. In particular, the instant invention features small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules and methods used to modulate the expression of BCL2 genes. A siNA of the invention can be unmodified or chemically-modified. A siNA of the instant invention can be chemically synthesized, expressed from a vector or enzymatically synthesized. The instant invention also features various chemically-

modified synthetic short interfering nucleic acid (siNA) molecules capable of modulating BCL2 gene expression or activity in cells by RNA interference (RNAi). The use of chemically-modified siNA improves various properties of native siNA molecules through increased resistance to nuclease degradation *in vivo* and/or through improved cellular uptake. Further, contrary to earlier published studies, siNA having multiple chemical modifications retains its RNAi activity. The siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, target validation, genomic discovery, genetic engineering, and pharmacogenomic applications.

In one embodiment, the invention features one or more siNA molecules and methods that independently or in combination modulate the expression of gene(s) encoding proteins, such as BCL2 proteins, associated with the maintenance and/or development of cancer and other proliferative diseases, such as genes encoding sequences comprising those sequences referred to by GenBank Accession Nos. shown in **Table I**, referred to herein generally as BCL2. The description below of the various aspects and embodiments is provided with reference to the exemplary BCL2 protein, including components or subunits thereof. However, the various aspects and embodiments are also directed to other genes which express other BCL2 or BCL2-related proteins, such as BCL-XL, BCL2-L1, MCL-1 CED-9, BAG-1, E1B-194 and BCL-A1, all referred to herein as BCL2. The various aspects and embodiments are also directed to other genes that are involved in BCL2 mediated pathways of signal transduction or gene expression that are involved in the progression, development, or maintenance of disease (e.g., cancer). Those additional genes can be analyzed for target sites using the methods described for BCL2s herein. Thus, the inhibition and the effects of such inhibition of the other genes can be performed as described herein.

In one embodiment, the invention features a siNA molecule that down-regulates expression of a BCL2 gene, for example, wherein the BCL2 gene comprises BCL2 encoding sequence.

In one embodiment, the invention features a siNA molecule having RNAi activity against BCL2 RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having BCL2 or other BCL2 encoding sequence, such as those sequences

having GenBank Accession Nos. shown in **Table I**. Chemical modifications as shown in **Tables III and IV** or otherwise described herein can be applied to any siNA construct of the invention.

In one embodiment, the invention features a siNA molecule having RNAi activity against BCL2 RNA, wherein the siNA molecule comprises a sequence complementary to any RNA having BCL2 encoding sequence, such as those sequences having BCL2 GenBank Accession Nos. shown in **Table I**. Chemical modifications as shown in **Tables III and IV** or otherwise described herein can be applied to any siNA construct of the invention.

In another embodiment, the invention features a siNA molecule having RNAi activity against a BCL2 gene, wherein the siNA molecule comprises nucleotide sequence complementary to nucleotide sequence of a BCL2 gene, such as those BCL2 sequences having GenBank Accession Nos. shown in **Table I**. In another embodiment, a siNA molecule of the invention includes nucleotide sequence that can interact with nucleotide sequence of a BCL2 gene and thereby mediate silencing of BCL2 gene expression, for example, wherein the siNA mediates regulation of BCL2 gene expression by cellular processes that modulate the chromatin structure of the BCL2 gene and prevent transcription of the BCL2 gene.

In another embodiment, the invention features a siNA molecule comprising nucleotide sequence, for example, nucleotide sequence in the antisense region of the siNA molecule that is complementary to a nucleotide sequence or portion of sequence of a BCL2 gene. In another embodiment, the invention features a siNA molecule comprising a region, for example, the antisense region of the siNA construct, complementary to a sequence or portion of sequence comprising a BCL2 gene sequence.

In one embodiment, the antisense region of BCL2 siNA constructs can comprise a sequence complementary to sequence having any of SEQ ID NOs. 1-414 or 829-832. The antisense region can also comprise sequence having any of SEQ ID NOs. 415-828, 837-840, 845-848, 853-856, 873, 875, 877, 879, 881 or 882. In another embodiment, the sense region of BCL2 constructs can comprise sequence having any of SEQ ID NOs. 1-414, 829-836, 841-844, 849-852, 872, 874, 876, 878 or 880. The sense region can comprise a sequence of SEQ ID NO. 861 and the antisense region can comprise a

sequence of SEQ ID NO. 862. The sense region can comprise a sequence of SEQ ID NO. 863 and the antisense region can comprise a sequence of SEQ ID NO. 864. The sense region can comprise a sequence of SEQ ID NO. 865 and the antisense region can comprise a sequence of SEQ ID NO. 866. The sense region can comprise a sequence of SEQ ID NO. 867 and the antisense region can comprise a sequence of SEQ ID NO. 868. The sense region can comprise a sequence of SEQ ID NO. 869 and the antisense region can comprise a sequence of SEQ ID NO. 870. The sense region can comprise a sequence of SEQ ID NO. 867 and the antisense region can comprise a sequence of SEQ ID NO. 871.

In one embodiment, a siNA molecule of the invention comprises any of SEQ ID NOs. 1-882. The sequences shown in SEQ ID NOs: 1-882 are not limiting. A siNA molecule of the invention can comprise any contiguous BCL2 sequence (e.g., about 19 to about 25, or about 19, 20, 21, 22, 23, 24 or 25 contiguous BCL2 nucleotides).

In yet another embodiment, the invention features a siNA molecule comprising a sequence, for example, the antisense sequence of the siNA construct, complementary to a sequence or portion of sequence comprising sequence represented by GenBank Accession Nos. shown in **Table I**. Chemical modifications in **Tables III and IV** and descrbed herein can be applied to any siRNA costruct of the invention.

In one embodiment of the invention a siNA molecule comprises an antisense strand having about 19 to about 29 nucleotides, wherein the antisense strand is complementary to a RNA sequence encoding a BCL2 protein, and wherein said siNA further comprises a sense strand having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) nucleotides, and wherein said sense strand and said antisense strand are distinct nucleotide sequences with at least about 19 complementary nucleotides.

In another embodiment of the invention a siNA molecule of the invention comprises an antisense region having about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28 or 29) nucleotides, wherein the antisense region is complementary to a RNA sequence encoding a BCL2 protein, and wherein said siNA further comprises a sense region having about 19 to about 29 nucleotides, wherein said sense region and said

antisense region comprise a linear molecule with at least about 19 complementary nucleotides.

In one embodiment of the invention a siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a BCL2 protein. The siNA further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a BCL2 gene or a portion thereof.

In another embodiment, a siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a BCL2 protein. The siNA molecule further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a BCL2 gene or a portion thereof.

In one embodiment, a siNA molecule of the invention has RNAi activity that modulates expression of RNA encoded by a BCL2 gene. Because BCL2 genes can share some degree of sequence homology with each other, siNA molecules can be designed to target a class of BCL2 genes (and associated receptor or ligand genes) or alternately specific BCL2 genes by selecting sequences that are either shared amongst different BCL2 targets or alternatively that are unique for a specific BCL2 target. Therefore, in one embodiment, the siNA molecule can be designed to target conserved regions of BCL2 RNA sequence having homology between several BCL2 receptor genes so as to target several BCL2 genes (e.g., different BCL2 isoforms, splice variants, mutant genes etc.) with one siNA molecule. In another embodiment, the siNA molecule can be designed to target a sequence that is unique to a specific BCL2 RNA sequence due to the high degree of specificity that the siNA molecule requires to mediate RNAi activity.

In one embodiment, nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are double-stranded nucleic acid molecules. In another embodiment, the siNA molecules of the invention consist of duplexes containing about 19 base pairs between oligonucleotides comprising about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24 or 25) nucleotides. In yet another embodiment, siNA molecules of the invention comprise duplexes with overhanging ends of about about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotides, for example, about 21-

nucleotide duplexes with about 19 base pairs and 3'-terminal mononucleotide, dinucleotide, or trinucleotide overhangs.

In one embodiment, the invention features one or more chemically-modified siNA constructs having specificity for BCL2 expressing nucleic acid molecules, such as RNA encoding a BCL2 protein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-deoxyribonucleotides, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications, when used in various siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish *et al., supra,* applicant demonstrates that multiple (greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise about 5% to 100% modified nucleotides (e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% modified nucleotides). The actual percentage of modified nucleotides present in a given siNA molecule will depend on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands.

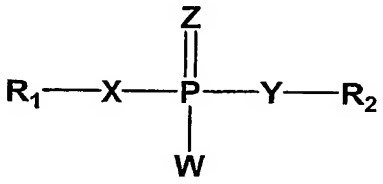
In a non-limiting example, the introduction of chemically-modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically-modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically-modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically-modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to an all-RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siNA, chemically-modified siNA can also minimize the possibility of activating interferon activity in humans.

The antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. The antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. The 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. The 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. The 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

One embodiment of the invention provides an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention in a manner that allows expression of the nucleic acid molecule. Another embodiment of the invention provides a mammalian cell comprising such an expression vector. The mammalian cell can be a human cell. The siNA molecule of the expression vector can comprise a sense region and an antisense region. The antisense region can comprise sequence complementary to a RNA or DNA sequence encoding BCL2 and the sense region can comprise sequence complementary to the antisense region. The siNA molecule can comprise two distinct strands having complementary sense and antisense

regions. The siNA molecule can comprise a single strand having complementary sense and antisense regions.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:

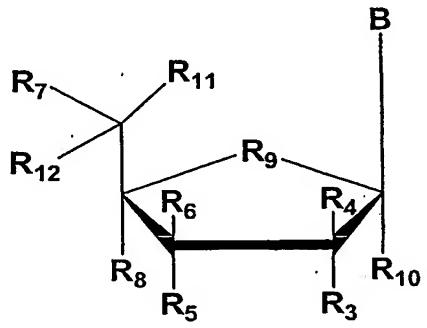


wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl, and wherein W, X, Y, and Z are optionally not all O.

The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7,. 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2,

3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:

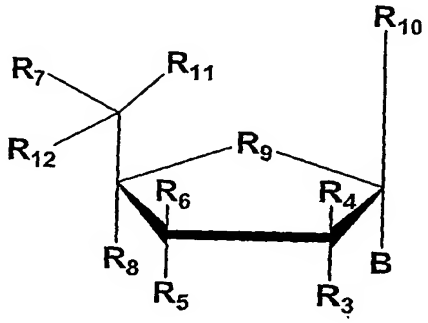


wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the

sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



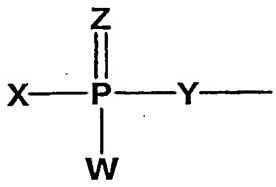
wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to

target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

In another embodiment, a siNA molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or alkylhalo; and wherein W, X, Y and Z are not all O.

In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*,

about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a siNA molecule, wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*,

about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and

optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about 1 to about 5, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages in each strand of the siNA molecule.

In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

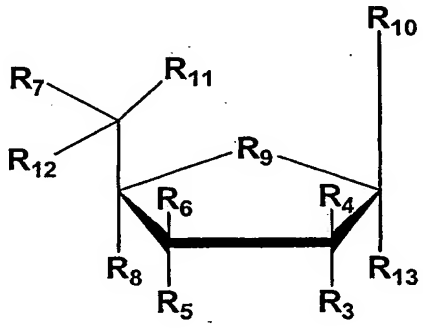
In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 18 to about 27 (*e.g.*, about 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27) nucleotides in length, wherein the duplex has about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary

chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (*e.g.*, about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (*e.g.*, about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

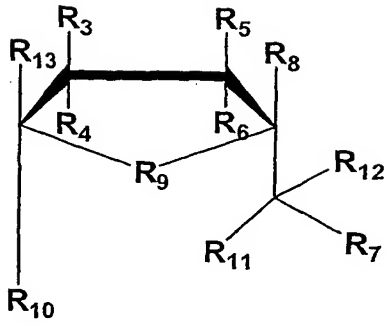
In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



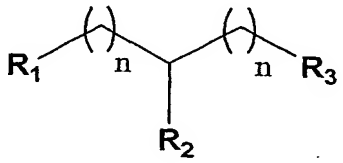
wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2.

In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention.

In another embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



wherein each n is independently an integer from 1 to 12, each R1, R2 and R3 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having Formula I, and R1, R2 or R3 serves as points of attachment to the siNA molecule of the invention.

In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, n = 1, and R3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in **Figure 10**).

In another embodiment, a moiety having any of Formula V, VI or VII of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, a moiety having Formula V, VI or VII can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

In another embodiment, a siNA molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula VI or VI is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In another embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a

plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or

alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine

nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 4 and 5** and **Tables III and IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where one or more purine nucleotides present in the sense region are purine ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides), and inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the siNA comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine

nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 4 and 5** and **Tables III and IV** herein.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemically-modified siNA comprises a sense region, where one or more pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and for example where one or more purine nucleotides present in the sense region are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (*e.g.*, wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides), and wherein inverted deoxy abasic modifications are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region, the sense region optionally further comprising a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides; and wherein the chemically-modified short interfering nucleic acid molecule comprises an antisense region, where one or more pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein one or more purine nucleotides present in the antisense region are selected from the group consisting

of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the antisense region optionally further comprising a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages.

In another embodiment, any modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2'-O,4'-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) against a BCL2 inside a cell or reconstituted *in vitro* system, wherein the chemical modification

comprises a conjugate covalently attached to the chemically-modified siNA molecule. In another embodiment, the conjugate is covalently attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, a conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a poly ethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese *et al.*, U.S. Serial No. 10/201,394, incorporated by reference herein. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of ≥ 2 nucleotides in length, for example 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural

setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. (See, for example, Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science,* 287, 820; and Jayasena, 1999, *Clinical Chemistry,* 45, 1628.)

In yet another embodiment, a non-nucleotide linker of the invention comprises abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g. polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, *18*:6353 and *Nucleic Acids Res.* 1987, *15*:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:5109; Ma *et al., Nucleic Acids Res.* 1993, *21*:2585 and *Biochemistry* 1993, *32*:1751; Durand *et al., Nucleic Acids Res.* 1990, *18*:6353; McCurdy *et al., Nucleosides & Nucleotides* 1991, *10*:287; Jschke *et al., Tetrahedron Lett.* 1993, *34*:301; Ono *et al., Biochemistry* 1991, *30*:9914; Arnold *et al.,* International Publication No. WO 89/02439; Usman *et al.,* International Publication No. WO 95/06731; Dudycz *et al.,* International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, *113*:4000, all hereby incorporated by reference herein. A "non-nucleotide" further means any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled

from two separate oligonucleotides do not comprise any ribonucleotides. All positions within the siNA can include chemically modified nucleotides and/or non-nucleotides such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-terminal phosphate group (e.g., a 2',3'-cyclic phosphate). In another embodiment, the single stranded siNA molecule of the invention comprises about 19 to about 29 nucleotides. In yet another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g.,

about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is

optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the siNA are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the antisense region are 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides), and a terminal cap modification, such as any modification described herein or shown in **Figure 10**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the siNA optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

In one embodiment, the invention features a method for modulating the expression of a BCL2 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the BCL2 gene in the cell.

In one embodiment, the invention features a method for modulating the expression of a BCL2 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 gene and wherein the sense strand sequence of the siNA comprises a sequence identical to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the BCL2 gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one BCL2 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 genes; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the BCL2 genes in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one BCL2 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 gene and wherein the sense strand sequence of the siNA comprises a sequence identical to the sequence of the target RNA; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the BCL2 genes in the cell.

In one embodiment, the invention features a method of modulating the expression of a BCL2 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under

conditions suitable to modulate the expression of the BCL2 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the BCL2 gene in that organism.

In one embodiment, the invention features a method of modulating the expression of a BCL2 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 gene and wherein the sense strand sequence of the siNA comprises a sequence identical to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the BCL2 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the BCL2 gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one BCL2 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the BCL2 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the BCL2 genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a BCL2 gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the BCL2 gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one BCL2 gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the BCL2 genes; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the BCL2 genes in the organism.

In one embodiment, the invention features a method for modulating the expression of a BCL2 gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the BCL2 gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the BCL2 gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one BCL2 gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the BCL2 gene; and (b) contacting the siNA molecule with a cell in vitro or in vivo under conditions suitable to modulate the expression of the BCL2 genes in the cell.

In one embodiment, the invention features a method of modulating the expression of a BCL2 gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the BCL2 gene; and (b) contacting the siNA molecule with a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the BCL2 gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the BCL2 gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one BCL2 gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA

comprises a single stranded sequence having complementarity to RNA of the BCL2 gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the BCL2 genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the BCL2 genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a BCL2 gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the BCL2 gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the BCL2 gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one BCL2 gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the BCL2 gene; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the BCL2 genes in the organism.

In one embodiment, the invention features a method of modulating the expression of a BCL2 gene in an organism comprising contacting the organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the BCL2 gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one BCL2 gene in an organism comprising contacting the organism with one or more siNA molecules of the invention under conditions suitable to modulate the expression of the BCL2 genes in the organism.

The siNA molecules of the invention can be designed to inhibit target (BCL2) gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a

target gene. Non-limiting examples of such RNAs include messenger RNA (mRNA), alternate RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

In another embodiment, the siNA molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families such as BCL2 family genes. As such, siNA molecules targeting multiple BCL2 targets can provide increased therapeutic effect. In addition, siNA can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example, the progression and/or maintenance of cancer.

In one embodiment, siNA molecule(s) and/or methods of the invention are used to inhibit the expression of gene(s) that encode RNA referred to by Genbank Accession, for example BCL2 genes encoding RNA sequence(s) referred to herein by Genbank Accession number, for example Genbank Accession Nos. shown in **Table I.**

In one embodiment, the invention features a method comprising: (a) generating a library of siNA constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example, about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of 4^N, where N represents the number of base paired nucleotides in each of the siNA construct strands (*eg.* for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 4^{19}); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target BCL2 RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described in Example 7 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of BCL2 RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target BCL2 RNA sequence. The target BCL2 RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of siNA molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one embodiment, the siNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising siNA molecules of the invention, which can be chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for treating or preventing a disease or condition in a subject, comprising administering to the subject

a composition of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In yet another embodiment, the invention features a method for reducing or preventing tissue rejection in a subject comprising administering to the subject a composition of the invention under conditions suitable for the reduction or prevention of tissue rejection in the subject.

In another embodiment, the invention features a method for validating a BCL2 gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a BCL2 target gene; (b) introducing the siNA molecule into a cell, tissue, or organism under conditions suitable for modulating expression of the BCL2 target gene in the cell, tissue, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, or organism.

In another embodiment, the invention features a method for validating a BCL2 target comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a BCL2 target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the BCL2 target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, insect, bacterial, viral or other sources, wherein the system comprises the components required for RNAi acitivity. The term "biological system" includes, for example, a cell, tissue, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid molecule of the invention (e.g., siNA). Such detectable changes include, but are not limited to, changes in shape, size, proliferation, motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change

can also include expression of reporter genes/molecules such as Green Florescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a BCL2 target gene in a cell, tissue, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one BCL2 target gene in a cell, tissue, or organism.

In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

In one embodiment, the synthesis of a siNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

In one embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second oligonucleotide sequence strand further comprises a chemical moiety than can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker

molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions using an alkylamine base such as methylamine. In one embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example, using acidic conditions.

In a further embodiment, the method for siNA synthesis is a solution phase synthesis or hybrid phase synthesis wherein both strands of the siNA duplex are synthesized in tandem using a cleavable linker attached to the first sequence which acts a scaffold for synthesis of the second sequence. Cleavage of the linker under conditions suitable for hybridization of the separate siNA sequence strands results in formation of the double-stranded siNA molecule.

In another embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing one oligonucleotide sequence strand of the siNA molecule, wherein the sequence comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of another oligonucleotide sequence; (b) synthesizing a second oligonucleotide sequence having complementarity to the first sequence strand on the scaffold of (a), wherein the second sequence comprises the other strand of the double-stranded siNA molecule and wherein the second sequence further comprises a chemical moiety than can be used to isolate the attached oligonucleotide sequence; (c) purifying the product of (b) utilizing the chemical moiety of the second oligonucleotide sequence strand under conditions suitable for isolating the full-length sequence comprising both siNA oligonucleotide strands connected by the cleavable linker and under conditions suitable for the two siNA oligonucleotide strands to

hybridize and form a stable duplex. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions. In another embodiment, cleavage of the linker molecule in (c) above takes place after deprotection of the oligonucleotide. In another embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity or differing reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place either concomitantly or sequentially. In one embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group.

In another embodiment, the invention features a method for making a double-stranded siNA molecule in a single synthetic process comprising: (a) synthesizing an oligonucleotide having a first and a second sequence, wherein the first sequence is complementary to the second sequence, and the first oligonucleotide sequence is linked to the second sequence via a cleavable linker, and wherein a terminal 5'-protecting group, for example, a 5'-O-dimethoxytrityl group (5'-O-DMT) remains on the oligonucleotide having the second sequence; (b) deprotecting the oligonucleotide whereby the deprotection results in the cleavage of the linker joining the two oligonucleotide sequences; and (c) purifying the product of (b) under conditions suitable for isolating the double-stranded siNA molecule, for example using a trityl-on synthesis strategy as described herein.

In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scaringe *et al.*, US Patent Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of Formulae

I-VII or any combination thereof that increases the nuclease resistance of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased nuclease resistance comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased nuclease resistance.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the sense and antisense strands of the siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the sense and antisense strands of the siNA molecule.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for

isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA construct.

In another embodiment, the invention features a method for generating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to a chemically-modified siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA molecule.

In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi against a BCL2 in a cell, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule and/or proteins or other factors that are essential for RNAi in a manner that would decrease the efficacy of RNAi mediated by such siNA constructs.

In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against BCL2 comprising (a) introducing

nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a BCL2 target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target RNA.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a BCL2 target DNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target DNA.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules against BCL2 with improved cellular uptake comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

In one embodiment, the invention features siNA constructs that mediate RNAi against a BCL2, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example, by attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese *et al.*, U.S. Serial No. 10/201,394 incorporated by reference herein.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability, comprising (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such conjugates can include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; polyamines, such as spermine or spermidine; and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include the siNA and a vehicle that promotes introduction of the siNA. Such a kit can also include instructions to allow a user of the kit to practice the invention.

The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner; see for example Bass, 2001, *Nature,* 411, 428-429; Elbashir *et al.,* 2001, *Nature,* 411, 494-498; and Kreutzer *et al.,* International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.,* International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.,* International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.,* International PCT Publication No. WO 00/44914; Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237; Hutvagner and Zamore, 2002, *Science,* 297, 2056-60; McManus *et al.,* 2002, *RNA,* 8, 842-850; Reinhart *et al.,* 2002, *Gene & Dev.,* 16, 1616-1626; and Reinhart & Bartel, 2002, *Science,* 297, 1831). Non limiting examples of siNA molecules of the invention are shown in **Figures 4-6,** and **Tables II, III, and IV** herein. For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand); the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a hairpin secondary structure, having self-complementary sense and antisense regions,

wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.*, 2002, *Cell.*, 110, 563-574 and Schwarz *et al.*, 2002, *Molecular Cell*, 10, 537-568), or 5',3'-diphosphate. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30,

40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, or epigenetics. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure to alter gene expression (see, for example, Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237).

By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit" it is meant that the activity of a gene expression product or level of RNAs or equivalent RNAs encoding one or more gene products is reduced below that observed in the absence of the nucleic acid molecule of the invention. In one embodiment, inhibition with a siNA molecule preferably is below that level observed in the presence of an inactive or attenuated molecule that is unable to mediate an RNAi response. In another embodiment, inhibition of gene expression with the siNA molecule of the instant invention is greater in the presence of the siNA molecule than in its absence.

By "gene" or "target gene" is meant, a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts.

By "BCL2" as used herein is meant, any protein, peptide, polypeptide, and/or polynucleotide having BCL2 or BCL2 family (eg. BCL2, BCL-XL, BCL2-L1, MCL-1 CED-9, BAG-1, E1B-194 or A1) activity or generated by BCL2 translocation. In a non-limiting example, BCL2 can be used to describe polynucleotides referred to by Genbank Accession number in **Table I** or any other BCL2 encoding nucleic acid sequence.

By "BCL2 protein" is meant, any BCL2 or BCL2 family peptide or protein or a component thereof, wherein the peptide or protein is encoded by a BCL2 gene or BCL2 activity.

By "highly conserved sequence region" is meant, a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner *et al.*, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.*, 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner *et al.*, 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The siRNA molecules of the invention represent a novel therapeutic approach to treat a variety of pathologic indications or other conditions, such as cancer, including but not limited to ovarian cancer, malignant melanoma, multiple myeloma, non-small cell lung cancer, prostate cancer, including malignant blood diseases such as lymphomas (eg. non-Hodgkins and Hodgkins lymphomas, and mantle cell lymphoma) leukemias (eg. chronic myeloid leukemia, CML; acute myeloid leukemias, AML; secondary leukemias, acute lymphoblastic leukemias, ALL; chronic lymphoid leukemia; CLL), polycytemia vera, idiopathic myelofibrosis, essential thrombocythemia, myelodysplastic syndromes, autoimmune disease (eg. multiple sclerosis, lupus, rheumatoid arthritis, insulin dependent diabetes, encephalitis, Rasmussen's encephalitis, thyroiditis, Crohn's disease, fibromyalgia, Grave's disease, Guillain Barre syndrome, chronic fatigue syndrome, autoimmune hepatitis, Meniere's disease, Myasthenia Gravis, cardiomyopathy, polymyalgia, Psoriasis, ulcerative collitis, etc.) viral infection (eg. HIV, HCV, HBV, RSV, CMV, HSV, influenza, rhinovirus etc.) and any other diseases or conditions that are related to the levels of BCL2 in a cell or tissue, alone or in combination with other therapies. The reduction of BCL2 expression (specifically BCL2 RNA levels) and thus reduction in the level of the respective protein relieves, to some extent, the symptoms of the disease or condition.

In one embodiment of the present invention, each sequence of a siNA molecule of the invention is independently about 18 to about 24 nucleotides in length, in specific embodiments about 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 17 to about 23 base pairs (*e.g.*, about 17, 18, 19, 20, 21, 22 or 23). In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (*e.g.*, about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (*e.g.*, 38, 39, 40, 41, 42, 43 or 44) nucleotides in length and comprising about 16 to about 22 (*e.g.*, about 16, 17, 18, 19, 20, 21 or 22) base pairs. Exemplary siNA molecules of the invention are shown in **Table II**. Exemplary synthetic siNA molecules of the invention are shown in **Tables III and IV** and/or **Figures 4-5**.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

The siNA molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in **Tables II-III** and/or **Figures 4-5**. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in **Table IV** can be applied to any siNA sequence of the invention.

In another aspect, the invention provides mammalian cells containing one or more siNA molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribo-furanose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. In one embodiment, a subject is a mammal or mammalian cells. In another embodiment, a subject is a human or human cells.

The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research,* 29, 2437-2447).

The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed herein (e.g., cancers and othe proliferative conditions). For example, to treat a particular disease or condition, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the siNA molecules can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents to treat a disease or condition. Non-limiting examples of other therapeutic agents that can be readily combined with a siNA molecule of the invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

In one embodiment, the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the invention, in a manner which allows expression of the siNA molecule. For example, the vector can contain sequence(s) encoding both strands of a siNA molecule comprising a duplex. The vector can also contain sequence(s) encoding a single nucleic acid molecule that is self-complementary and thus forms a siNA molecule. Non-limiting examples of such expression vectors are described in Paul *et al.*, 2002, *Nature Biotechnology,* 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology,* 19, 497; Lee *et al.*, 2002, *Nature Biotechnology,* 19, 500; and Novina *et al.*, 2002, *Nature Medicine*, advance online publication doi:10.1038/nm725.

In another embodiment, the invention features a mammalian cell, for example, a human cell, including an expression vector of the invention.

In yet another embodiment, the expression vector of the invention comprises a sequence for a siNA molecule having complementarity to a RNA molecule referred to by a Genbank Accession numbers, for example Genbank Accession Nos. shown in **Table I.**

In one embodiment, an expression vector of the invention comprises a nucleic acid sequence encoding two or more siNA molecules, which can be the same or different.

In another aspect of the invention, siNA molecules that interact with target RNA molecules and down-regulate gene encoding target RNA molecules (for example target RNA molecules referred to by Genbank Accession numbers herein) are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecules bind and down-regulate gene function or expression via RNA interference (RNAi). Delivery of siNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a non-limiting example of a scheme for the synthesis of siNA molecules. The complementary siNA sequence strands, strand 1 and strand 2, are synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis. The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on the terminal nucleotide of the tandem oligonucleotide. Upon cleavage

and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the properties of the terminal protecting group, for example by applying a trityl on purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

Figure 2 shows a MALDI-TOV mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the siNA duplex generated from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

Figure 3 shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for example viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be introduced directly into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

Figure 4A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs.

Figure 4A: The sense strand comprises 21 nucleotides having four phosphorothioate 5'- and 3'-terminal internucleotide linkages, wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21

nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and four 5'-terminal phosphorothioate internucleotide linkages and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4B: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4C: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4D: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and

wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4E: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 4F: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro

modified nucleotides and all purine nucleotides that may be present are 2'-deoxy nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand of constructs A-F comprise sequence complementary to any target nucleic acid sequence of the invention.

Figure 5A-F shows non-limiting examples of specific chemically-modified siNA sequences of the invention. **A-F** applies the chemical modifications described in **Figure 4A-F** to a BCL2 siNA sequence.

Figure 6 shows non-limiting examples of different siNA constructs of the invention. The examples shown (constructs 1, 2, and 3) have 19 representative base pairs; however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example comprising about 1, 2, 3, or 4 nucleotides in length, preferably about 2 nucleotides. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 *in vivo* and/or *in vitro*. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 *in vivo* and/or *in vitro*, which can optionally utilize another biodegradable linker to generate the active siNA construct 1 *in vivo* and/or *in vitro*. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use *in vivo* or *in vitro* and/or *in vitro*.

Figure 7A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate siNA hairpin constructs.

Figure 7A: A DNA oligomer is synthesized with a 5'-restriction site (R1) sequence followed by a region having sequence identical (sense region of siNA) to a predetermined BCL2 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, which is followed by a loop sequence of defined sequence (X), comprising, for example, about 3 to about 10 nucleotides.

Figure 7B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence that will result in a siNA transcript having specificity for a BCL2 target sequence and having self-complementary sense and antisense regions.

Figure 7C: The construct is heated (for example to about 95°C) to linearize the sequence, thus allowing extension of a complementary second DNA strand using a primer to the 3'-restriction sequence of the first strand. The double-stranded DNA is then inserted into an appropriate vector for expression in cells. The construct can be designed such that a 3'-terminal nucleotide overhang results from the transcription, for example by engineering restriction sites and/or utilizing a poly-U termination region as described in Paul *et al.*, 2002, *Nature Biotechnology,* 29, 505-508.

Figure 8A-C is a diagrammatic representation of a scheme utilized in generating an expression cassette to generate double-stranded siNA constructs.

Figure 8A: A DNA oligomer is synthesized with a 5'-restriction (R1) site sequence followed by a region having sequence identical (sense region of siNA) to a predetermined BCL2 target sequence, wherein the sense region comprises, for example, about 19, 20, 21, or 22 nucleotides (N) in length, and which is followed by a 3'-restriction site (R2) which is adjacent to a loop sequence of defined sequence (X).

Figure 8B: The synthetic construct is then extended by DNA polymerase to generate a hairpin structure having self-complementary sequence.

Figure 8C: The construct is processed by restriction enzymes specific to R1 and R2 to generate a double-stranded DNA which is then inserted into an appropriate vector for expression in cells. The transcription cassette is designed such that a U6 promoter region flanks each side of the dsDNA which generates the separate sense and antisense strands of the siNA. Poly T termination sequences can be added to the constructs to generate U overhangs in the resulting transcript.

Figure 9A-E is a diagrammatic representation of a method used to determine target sites for siNA mediated RNAi within a particular target nucleic acid sequence, such as messenger RNA.

Figure 9A: A pool of siNA oligonucleotides are synthesized wherein the antisense region of the siNA constructs has complementarity to target sites across the target nucleic acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siNA.

Figure 9B&C: **(Figure 9B)** The sequences are pooled and are inserted into vectors such that **(Figure 9C)** transfection of a vector into cells results in the expression of the siNA.

Figure 9D: Cells are sorted based on phenotypic change that is associated with modulation of the target nucleic acid sequence.

Figure 9E: The siNA is isolated from the sorted cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

Figure 10 shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including **(1)** [3-3']-inverted deoxyribose; **(2)** deoxyribonucleotide; **(3)** [5'-3']-3'-deoxyribonucleotide; **(4)** [5'-3']-ribonucleotide; **(5)** [5'-3']-3'-O-methyl ribonucleotide; **(6)** 3'-glyceryl; **(7)** [3'-5']-3'-deoxyribonucleotide; **(8)** [3'-3']-deoxyribonucleotide; **(9)** [5'-2']-deoxyribonucleotide; and **(10)** [5-3']-dideoxyribonucleotide. In addition to modified and unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any combination thereof.

Figure 11 shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistance while preserving the ability to mediate RNAi activity. Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g. introducing 2'-mofications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct in tested in an appropriate system (e.g. human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA

construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

Figure 12 shows a non-limiting example of reduction of BCL2 mRNA in A549 cells mediated by chemically-modified siNAs that target BCL2 mRNA. A549 cells were transfected with 0.25 ug/well of lipid complexed with 25 nM siNA. A siNA construct comprising ribonucleotides and 3'-terminal dithymidine caps (RPI#30998/31074) was tested along with a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro pyrimidine nucleotides and purine ribonucleotides in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxyabasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage (RPI#31368/31369), which was also compared to a matched chemistry inverted control (RPI#31370/31371) and a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro pyrimidine and 2'-deoxy-2'-fluoro purine nucleotides in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxyabasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage (RPI#31372/31373) which was also compared to a matched chemistry inverted control (RPI#31374/31375). In addition, the siNA constructs were also compared to untreated cells, cells transfected with lipid and scrambled siNA constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control). As shown in the figure, the siNA constructs show significant reduction of BCL2 RNA expression compared to scrambled, untreated, and transfection controls.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of action of Nucleic Acid Molecules of the Invention

The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability

and activity *in vivo*; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured *in vitro* and/or *in vivo* where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased *in vitro* and/or *in vivo* compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature,* 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, 2001, *Nature,* 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in

translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably though cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science*, 297, 1818-1819; Volpe *et al.*, 2002, *Science*, 297, 1833-1837; Jenuwein, 2002, *Science*, 297, 2215-2218; and Hall *et al.*, 2002, *Science*, 297, 2232-2237). As such, siNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA

activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell,* 107, 309); however, siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (*e.g.*, certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides or 2'-deoxy-2'-fluoro nucleotides. **Table V** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl

residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman *et al.,* 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.,* 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.,* 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.,* 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. **Table V** outlines the amounts and the contact times of the reagents used in the synthesis

cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 μL of a solution of 1.5 mL N-methylpyrrolidinone, 750 μL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a

solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 min. The vial is brought to rt. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

The average stepwise coupling yields are typically >98% (Wincott *et al.,* 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.,* 1992, *Science* 256, 9923; Draper *et al.,* International PCT publication No. WO 93/23569; Shabarova *et al.,* 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.,* 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.,* 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al., supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.,* Eckstein *et al.,* International Publication No. WO 92/07065; Perrault *et al.,* 1990 *Nature* 344, 565; Pieken *et al.,* 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.,* International Publication No. WO 93/15187; and Rossi *et al.,* International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.,* U.S. Pat. No. 6,300,074; and Burgin *et al., supra;* all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.,* 1996, *Biochemistry*, 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al., International Publication* PCT No. WO 92/07065; Perrault *et al. Nature*, 1990, *344*, 565-568; Pieken *et al. Science,* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* , 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *U.S. Pat.* No. 5,334,711 and Beigelman *et al.,* 1995, *J. Biol. Chem.,* 270, 25702; Beigelman *et al.,* International PCT publication No. WO 97/26270; Beigelman *et al.,* U.S. Pat. No. 5,716,824; Usman *et al.,* U.S. Pat. No. 5,627,053; Woolf *et al.,* International PCT Publication No. WO 98/13526; Thompson *et al.,* USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.,* 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina *et al.,* 1997, *Bioorg. Med. Chem.,* 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi is cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.*, International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the

invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Therapeutic nucleic acid molecules (*e.g.,* siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered.

Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.,* multiple siNA molecules targeted to different genes; nucleic acid molecules

coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of subjects with siNA molecules can also include combinations of different types of nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'- cap structure, for example on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic *et al.*, U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap is selected from the group consisting of glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety.

In non-limiting examples, the 3'-cap is selected from the group consisting of glyceryl, inverted deoxy abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide;

phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The term "alkyl" also includes alkynyl groups that have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino or SH.

Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090;

Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417, and Mesmaeker *et al.,* 1994, *Novel Backbone Replacements for Oligonucleotides,* in *Carbohydrate Modifications in Antisense Research,* ACS, 24-39.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic *et al.,* U.S. Pat. No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.,* U.S. Pat. No. 5,672,695 and Matulic-Adamic *et al.,* U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro,* stability, and ease of introduction of such oligonucleotides to the target site, *e.g.,* to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

A siNA molecule of the invention can be adapted for use to treat cancer, including but not limited to ovarian cancer, malignant melanoma, multiple myeloma, non-small cell lung cancer, prostate cancer, including malignant blood diseases such as lymphomas (eg. non-Hodgkins and Hodgkins lymphomas, and mantle cell lymphoma) leukemias (eg. chronic myeloid leukemia, CML; acute myeloid leukemias, AML; secondary leukemias, acute lymphoblastic leukemias, ALL; chronic lymphoid leukemia; CLL), polycytemia vera, idiopathic myelofibrosis, essential thrombocythemia, myelodysplastic syndromes, autoimmune disease (eg. multiple sclerosis, lupus, rheumatoid arthritis, insulin dependent diabetes, encephalitis, Rasmussen's encephalitis, thyroiditis, Crohn's disease, fibromyalgia, Grave's disease, Guillain Barre syndrome, chronic fatigue syndrome, autoimmune hepatitis, Meniere's disease, Myasthenia Gravis, cardiomyopathy, polymyalgia, Psoriasis, ulcerative collitis, etc.) viral infection (eg. HIV, HCV, HBV, RSV, CMV, HSV, influenza, rhinovirus etc.) and any other diseases or conditions that are related to the levels of BCL2 in a cell or tissue, alone or in combination with other therapies. For example, a siNA molecule can comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.*, 1999, *Mol. Membr. Biol.*, 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee *et al.*, 2000, *ACS Symp. Ser.*, 752, 184-192, all of which are incorporated herein by reference. Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins (see for example Gonzalez *et al.*, 1999, *Bioconjugate Chem.*, 10, 1068-1074), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid

molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry *et al.*, 1999, *Clin. Cancer Res.*, 5, 2330-2337 and Barry *et al.*, International PCT Publication No. WO 99/31262. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention can also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cells producing excess BCL2.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58) (Alkermes, Inc. Cambridge, MA); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al.*,1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.,* International PCT Publication No. WO 96/10391; Ansell *et al.,* International PCT Publication No. WO 96/10390; Holland *et al.,* International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the

physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained

action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already

mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either

be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the invention comprises compositions suitable for administering nucleic acid molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example,

triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell,* 22, 611-620; Connolly *et al.,* 1982, *J. Biol. Chem.,* 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.,* 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.,* 1981, *J. Med. Chem.,* 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavialability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese *et al.,* USSN 10/201,394, filed August 13, 2001; and Matulic-Adamic *et al.,* USSN 60/362,016, filed March 6, 2002.

Alternatively, certain siNA molecules of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.,* Izant and Weintraub, 1985, *Science,* 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci.,* USA 83, 399; Scanlon *et al.,* 1991, *Proc. Natl. Acad. Sci. USA,* 88, 10591-5; Kashani-Sabet *et al.,* 1992, *Antisense Res. Dev.,* 2, 3-15; Dropulic *et al.,* 1992, *J. Virol.,* 66, 1432-41; Weerasinghe *et al.,* 1991, *J. Virol.,* 65, 5531-4; Ojwang *et al.,* 1992, *Proc. Natl. Acad. Sci. USA,* 89, 10802-6; Chen *et al.,* 1992, *Nucleic Acids Res.,* 20, 4581-9; Sarver *et al.,* 1990 *Science,* 247, 1222-1225; Thompson *et al.,* 1995, *Nucleic Acids Res.,* 23, 2259; Good *et al.,* 1997, *Gene Therapy,* 4, 45. Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a enzymatic nucleic acid (Draper *et al.,* PCT WO 93/23569, and Sullivan *et al.,* PCT WO 94/02595; Ohkawa *et al.,* 1992, *Nucleic Acids Symp. Ser.,* 27, 15-6; Taira *et al.,* 1991, *Nucleic Acids Res.,* 19, 5125-30; Ventura *et al.,* 1993, *Nucleic Acids Res.,* 21, 3249-55; Chowrira *et al.,* 1994, *J. Biol. Chem.,* 269, 25856.

In another aspect of the invention, RNA molecules of the present invention can be expressed from transcription units (see for example Couture *et al.,* 1996, *TIG.,* 12, 510) inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. In another embodiment, pol III based constructs are used to express nucleic acid molecules of the invention (see for example Thompson, U.S. Pats. Nos. 5,902,880 and 6,146,886). The recombinant vectors capable of expressing the siNA molecules can be delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the siNA molecule interacts with the target mRNA and generates an RNAi response. Delivery of siNA molecule expressing vectors can be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from a subject followed by reintroduction into the subject, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.,* 1996, *TIG.,* 12, 510).

In one aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one siNA molecule of the instant invention. The expression vector can encode one or both strands of a siNA duplex, or a single self-complementary strand that self hybridizes into a siNA duplex. The nucleic acid sequences encoding the siNA molecules of the instant invention can be operably linked in a manner that allows expression of the siNA molecule (see for example Paul *et al.,* 2002, *Nature Biotechnology,* 19, 505; Miyagishi and Taira, 2002, *Nature Biotechnology,* 19, 497; Lee *et al.,* 2002, *Nature Biotechnology,* 19, 500; and Novina *et al.,* 2002, *Nature Medicine,* advance online publication doi:10.1038/nm725).

In another aspect, the invention features an expression vector comprising: a) a transcription initiation region (*e.g.,* eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.,* eukaryotic pol I, II or III termination region); and c) a nucleic acid sequence encoding at least one of the siNA molecules of the instant invention; wherein said sequence is operably linked to said initiation region and said termination region, in a manner that allows expression and/or delivery of the siNA molecule. The vector can optionally include an open reading frame (ORF) for a protein

operably linked on the 5' side or the 3'-side of the sequence encoding the siNA of the invention; and/or an intron (intervening sequences).

Transcription of the siNA molecule sequences can be driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters are expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type depends on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber *et al.*, 1993, *Methods Enzymol.*, 217, 47-66; Zhou *et al.*, 1990, *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that nucleic acid molecules expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet *et al.*, 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang *et al.*, 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu *et al.*, 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992, *EMBO J.*, 11, 4411-8; Lisziewicz *et al.*, 1993, *Proc. Natl. Acad. Sci. U. S. A*, 90, 8000-4; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as siNA in cells (Thompson *et al., supra*; Couture and Stinchcomb, *1996, supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, U.S. Pat. No. 5,624,803; Good *et al.*, 1997, *Gene Ther.*, 4, 45; Beigelman *et al.*, International PCT Publication No. WO 96/18736. The above siNA transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra)*.

In another aspect the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the siNA molecules of the invention in a manner that allows expression of that siNA molecule. The expression vector comprises in one

embodiment; a) a transcription initiation region; b) a transcription termination region; and c) a nucleic acid sequence encoding at least one strand of the siNA molecule, wherein the sequence is operably linked to the initiation region and the termination region in a manner that allows expression and/or delivery of the siNA molecule.

In another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; and d) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the open reading frame and the termination region in a manner that allows expression and/or delivery of the siNA molecule. In yet another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; and d) a nucleic acid sequence encoding at least one siNA molecule, wherein the sequence is operably linked to the initiation region, the intron and the termination region in a manner which allows expression and/or delivery of the nucleic acid molecule.

In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) an open reading frame; and e) a nucleic acid sequence encoding at least one strand of a siNA molecule, wherein the sequence is operably linked to the 3'-end of the open reading frame and wherein the sequence is operably linked to the initiation region, the intron, the open reading frame and the termination region in a manner which allows expression and/or delivery of the siNA molecule.

BCL2 biology and biochemistry

The BCL2 family comprises both pro-apoptotic and anti-apoptotic members. The apoptotic antagonists include BCL2, Bcl-XL, Mcl-1 and A1, whereas Bax, Bak, Bad, Bcl-Xs Bcl-X-beta and Bik are pro-apoptotic members. BCL2 family members can possess at least one of four conserved motifs known as BCL2 homologous domains (BH1 to BH4). These proteins are believed to be membrane bound and their ability to undergo both homodimerization and heterodimerization has been proposed to regulate apoptosis.

The BCL2 gene is abnormally expressed in about 85% of follicular lymphomas and about 20% of diffuse lymphomas due to a t(14;18)(q32;q21) chromosomal rearrangement between the BCL2 locus on chromosome 18 and the immunoglobulin heavy chain locus on chromosome 14 (Yunis et al., 316 N. Engl. J. Med. 79, 1987). This chromosomal rearrangement represents the most common found in lymphoid malignancies in humans. A BCL2/IgH fusion message is expressed; however, the BCL2 protein-coding region is not interrupted since the major breakpoint region lies in the 3' non-translated region of the BCL2 transcript (Cleary et al., 47 Cell 19, 1986). The BCL2 gene represents a new form of proto-oncogene in that it encodes a mitochondrial protein which inhibits cell senescence (Hockenbery et al., 348 Nature 334, 1990), leading to extended survival of B cells transfected with this gene (Nunez et al., 86 Proc. Natl. Acad. Sci. USA 4589, 1989). Additionally, BCL2 over-expression may not always be caused by t(14;18), because it is often detected in lymphomas without BCL2 rearrangement. Recent studies have shown that increased expression of BCL2 can also result from BCL2 gene amplification in diffuse large B-cell lymphomas. Similarly, it has been speculated that the mutations of the open reading frame might cause increased expression of BCL2 by affecting the interactions of BCL2 with other proteins. BCL2 over-expression is implicated in several cancers, such as ovarian cancer, malignant melanoma, multiple myeloma, non-small cell lung cancer, prostate cancer, including malignant blood diseases, such as lymphomas (eg. non-Hodgkins and Hodgkins lymphomas, and mantle cell lymphoma), leukemias (eg. chronic myeloid leukemia, CML; acute myeloid leukemias, AML; secondary leukemias, acute lymphoblastic leukemias, ALL; chronic lymphoid leukemia; CLL), polycytemia vera, idiopathic myelofibrosis, essential thrombocythemia, and myelodysplastic syndromes.

At least three different forms of BCL2 mRNAs are found in pre-B cells and T cells, which vary due to alternative splicing and promoter usage. Two different proteins are produced, a 21 kD and a 26 kD peptide which vary at their carboxy-termini. Both forms have identical N termini encoded in exon 2 of the gene. Consequently, this region and others provide suitable targets for siRNA mediated RNA interference.

The use of small interfering nucleic acid molecules targeting BCL2 provides a class of novel therapeutic agents that can be used in the diagnosis and treatment of cancers or any other disease or condition that responds to modulation of BCL2 genes.

Examples:

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Tandem synthesis of siNA constructs

Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example, a succinyl-based linker. Tandem synthesis as described herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

After completing a tandem synthesis of a siNA oligo and its complement in which the 5'-terminal dimethoxytrityl (5'-O-DMT) group remains intact (trityl on synthesis), the oligonucleotides are deprotected as described above. Following deprotection, the siNA sequence strands are allowed to spontaneously hybridize. This hybridization yields a duplex in which one strand has retained the 5'-O-DMT group while the complementary strand comprises a terminal 5'-hydroxyl. The newly formed duplex behaves as a single molecule during routine solid-phase extraction purification (Trityl-On purification) even though only one molecule has a dimethoxytrityl group. Because the strands form a stable duplex, this dimethoxytrityl group (or an equivalent group, such as other trityl groups or other hydrophobic moieties) is all that is required to purify the pair of oligos, for example, by using a C18 cartridge.

Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see **Figure 1**) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexaflurorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting

oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M $NH_4H_2CO_3$.

Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H2O, and 2 CV 50mM NaOAc. The sample is loaded and then washed with 1 CV H2O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H2O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H20 followed by 1 CV 1M NaCl and additional H2O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

Figure 2 provides an example of MALDI-TOV mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA contract only shows a single peak. Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

Example 2: Identification of potential siNA target sites in any RNA sequence

The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as Genbank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with

other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using *in vitro* RNA cleavage assays, cell culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays to determine efficient reduction in target gene expression.

Example 3: Selection of siNA molecule target sites in a RNA

The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

1. The target sequence is parsed *in silico* into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.

2. In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can

identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.

3. In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.

4. The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.

5. The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.

6. The ranked siNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided whenever better sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.

7. The ranked siNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.

8. Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siNA duplex, while the reverse complement of the left 21

nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex (see **Tables II and III**). If terminal TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

9. The siNA molecules are screened in an *in vitro*, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.

In an alternate approach, a pool of siNA constructs specific to a BCL2 target sequence is used to screen for target sites in cells expressing BCL2 RNA, such as human T24, NHDF, HEK, HuVEC, 3t3-L1, or A549 cells. The general strategy used in this approach is shown in **Figure 9.** A non-limiting example of such as pool is a pool comprising sequences having sense sequences comprising SEQ ID NOs. 1-414, 829-836, 841-844, and 849-852 and antisense sequences comprising SEQ ID NOs. 415-828, 837-840, 845-848, and 853-856 respectively. Cells expressing BCL2 (e.g., A549) are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with BCL2 inhibition are sorted. The pool of siNA constructs can be expressed from transcription cassettes inserted into appropriate vectors (see for example **Figure 7** and **Figure 8**). The siNA from cells demonstrating a positive phenotypic change (e.g., decreased proliferation, decreased BCL2 mRNA levels or decreased BCL2 protein expression), are sequenced to determine the most suitable target site(s) within the target BCL2 RNA sequence.

Example 4: BCL2 targeted siNA design

siNA target sites were chosen by analyzing sequences of the BCL2 RNA target and optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), by using a library of siNA molecules as described in Example 3, or alternately by using an *in vitro* siNA system as described in Example 6 herein. siNA molecules were designed that could bind each target and are optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number

of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites within any known RNA sequence, for example those RNA sequences corresponding to the any gene transcript.

Chemically modified siNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g. liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantity RNAi activity. Synthetic siNA constructs that possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development (see for example **Figure 11**).

Example 5: Chemical Synthesis and Purification of siNA

siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can by synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman *et al.*, US Patent Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe *et al.*, US Patent Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N,N-diisopropylphosphoroamidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2′-O-Silyl Ethers can be used in conjunction with acid-labile 2′-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe *supra*. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman *et al.*, US Patent 5,631,360, incorporated by reference herein in its entirety).

During solid phase synthesis, each nucleotide is added sequentially (3′- to 5′-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5′-end of the first nucleoside. The support is then washed and any unreacted 5′-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5′-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5′-O-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Scaringe *supra*, Usman et al., US 5,831,071, US 6,353,098, US 6,437,117, and Bellon et al., US 6,054,576, US 6,162,909, US 6,303,773, herein incorporated by reference in their entirety. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has

observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35°C for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35°C for 30 minutes, TEA-HF is added and the reaction maintained at about 65°C for an additional 15 minutes.

Example 6: RNAi *in vitro* assay to assess siNA activity

An *in vitro* assay that recapitulates RNAi in a cell-free system is used to evaluate siNA constructs targeting BCL2 RNA targets. The assay comprises the system described by Tuschl *et al.*, 1999, *Genes and Development*, 13, 3191-3197 and Zamore *et al.*, 2000, *Cell*, 101, 25-33 adapted for use with BCL2 target RNA. A Drosophila extract derived from syncytial blastoderm is used to reconstitute RNAi activity *in vitro*. Target RNA is generated via *in vitro* transcription from an appropriate BCL2 expressing plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min. at 90°C followed by 1 hour at 37°C , then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The Drosophila lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10% [vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug.ml creatine phosphokinase, 100 um GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C for 10 minutes before adding RNA, then incubated at 25° C for an additional 60 minutes. Reactions are quenched with 4 volumes of 1.25 x Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR

analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

Alternately, internally-labeled target RNA for the assay is prepared by *in vitro* transcription in the presence of [alpha-^{32}P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-^{32}P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by Phosphor Imager® quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

In one embodiment, this assay is used to determine target sites the BCL2 RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the BCL2 RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 7: Nucleic acid inhibition of BCL2 target RNA *in vivo*

siNA molecules targeted to the huma BCL2 RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity *in vivo*, for example, using the following procedure. The target sequences and the nucleotide location within the BCL2 RNA are given in **Table II and III**.

Two formats are used to test the efficacy of siNAs targeting BCL2. First, the reagents are tested in cell culture using, for example, T24, NHDF, HEK, HuVEC, 3t3-L1, or A549 cells to determine the extent of RNA and protein inhibition. siNA reagents (*e.g.*; see **Tables II and III**) are selected against the BCL2 target as described herein. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to, for example, T24, NHDF, HEK, HuVEC, 3t3-L1, or A549 cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (*eg.*, ABI 7700 Taqman®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control

with the same overall length and chemistry, but randomly substituted at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

Delivery of siNA to Cells

Cells (e.g., T24, NHDF, HEK, HuVEC, 3t3-L1, or A549 cells) are seeded, for example, at $1x10^5$ cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siNA (final concentration, for example 20nM) and cationic lipid (e.g., final concentration 2μg/ml) are complexed in EGM basal media (Biowhittaker) at 37°C for 30 mins in polystyrene tubes. Following vortexing, the complexed siNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at $1x10^3$ in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

Taqman and Lightcycler quantification of mRNA

Total RNA is prepared from cells following siNA delivery, for example, using Qiagen RNA purification kits for 6-well or Rneasy extraction kits for 96-well assays. For Taqman analysis, dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 μl reactions consisting of 10 μl total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1X TaqMan PCR reaction buffer (PE-Applied Biosystems), 5.5 mM $MgCl_2$, 300 μM each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AmpliTaq Gold (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/rxn) and normalizing to ß-

actin or GAPDH mRNA in parallel TaqMan reactions. For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcyler. A standard curve is generated for each primer pair using control cRNA. Values are represented as relative expression to GAPDH in each sample.

Western blotting

Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, *Nucleic Acids Research,* 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4°C. Following washes, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 8: Models useful to evaluate the down-regulation of BCL2 gene expression

Cell Culture

There are numerous cell culture systems that can be used to analyze reduction of BCL2 levels either directly or indirectly by measuring downstream effects. For example, T24, NHDF, HEK, HuVEC, 3t3-L1, or A549 cells can be used in cell culture experiments to assess the efficacy of nucleic acid molecules of the invention. As such, T24, NHDF, HEK, HuVEC, 3t3-L1, or A549 cells treated with nucleic acid molecules of the invention (e.g., siNA) targeting BCL2 RNA would be expected to have decreased BCL2 expression capacity compared to matched control nucleic acid molecules having a scrambled or inactive sequence. In a non-limiting example, cells are cultured and BCL2 expression is quantified, for example, by time-resolved immunofluorometric assay.

BCL2 messenger-RNA expression is quantitated with RT-PCR in cultured cells. Untreated cells are compared to cells treated with siNA molecules transfected with a suitable reagent, for example, a cationic lipid such as lipofectamine, and BCL2 protein and RNA levels are quantitated. Dose response assays are then performed to establish dose dependent inhibition of BCL2 expression.

In several cell culture systems, cationic lipids have been shown to enhance the bioavailability of oligonucleotides to cells in culture (Bennet, *et al.*, 1992, *Mol. Pharmacology*, 41, 1023-1033). In one embodiment, siNA molecules of the invention are complexed with cationic lipids for cell culture experiments. siNA and cationic lipid mixtures are prepared in serum-free DMEM immediately prior to addition to the cells. DMEM plus additives are warmed to room temperature (about 20-25°C) and cationic lipid is added to the final desired concentration and the solution is vortexed briefly. siNA molecules are added to the final desired concentration and the solution is again vortexed briefly and incubated for 10 minutes at room temperature. In dose response experiments, the RNA/lipid complex is serially diluted into DMEM following the 10 minute incubation.

The effect of siRNA compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following 6 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods know in the art, for example Northern blot analysis, Ribonuclease protection assays, and/or RT-PCR.

T-24 Cells

The human transitional cell bladder carcinoma cell line T-24 is obtained from the American Type Culture Collection (ATCC) (Manassas, Va.). T-24 cells are routinely cultured in complete McCoy's 5A basal media supplemented with 10% fetal calf serum, penicillin 100 units per mL, and streptomycin 100 micrograms per mL. Cells are routinely passaged by trypsinization and dilution when they have reached 90% confluence. Cells are seeded into 96-well plates at a density of about 7000 cells/well for use in RT-PCR analysis. For Northern blotting or other analysis, cells can be seeded

onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 Cells

The human lung carcinoma cell line A549 is obtained from the American Type Culture Collection (ATCC) (Manassas, Va.). A549 cells are routinely cultured in DMEM basal media supplemented with 10% fetal calf serum, penicillin 100 units per mL, and streptomycin 100 micrograms per mL. Cells are routinely passaged by trypsinization and dilution when they have reached 90% confluence.

NHDF Cells

Human neonatal dermal fibroblast (NHDF) are obtained from the Clonetics Corporation (Walkersville Md.). NHDFs are routinely maintained in Fibroblast Growth Medium supplemented as recommended by the supplier. Cells are maintained for up to 10 passages as recommended by the supplier.

HEK Cells

Human embryonic keratinocytes (HEK) are obtained from the Clonetics Corporation (Walkersville Md.). HEKs were routinely maintained in Keratinocyte Growth Medium formulated as recommended by the supplier. Cells are routinely maintained for up to 10 passages as recommended by the supplier.

HuVEC Cells

The human umbilical vein endothilial cell line HuVEC are obtained from the American Type Culure Collection (Manassas, Va.). HuVEC cells are routinely cultured in EBM supplemented with SingleQuots supplements. Cells are routinely passaged by trypsinization and dilution when they reached 90% confluence. The cells are maintained for up to 15 passages. Cells are seeded into 96-well plates at a density of about 10000 cells/well for use in RT-PCR analysis. For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

3T3-L1 Cells

The mouse embryonic adipocyte-like cell line 3T3-L1 are obtained from the American Type Culure Collection (Manassas, Va.). 3T3-L1 cells are routinely cultured in DMEM, high glucose supplemented with 10% fetal calf serum. Cells are routinely passaged by trypsinization and dilution when they reached 80% confluence. Cells are seeded into 96-well plates at a density of 4000 cells/well for use in RT-PCR analysis. For Northern blotting or other analyses, cells can be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Animal Models

Evaluating the efficacy of anti-BCL2 agents in animal models is an important prerequisite to human clinical trials. As in cell culture models, the most BCL2 sensitive mouse tumor xenografts are those derived from human carcinoma cells that express high levels of BCL2 protein.

Investigators have shown that nude mice bearing human renal cell carcinoma (RCC) xenografts are sensitive to anti- BCL2 antisense compounds, resulting in a partial regression of tumor growth (Uchida *et al.,* 2001, *Molecular Urology.,* 5, 71-78). Expression of BCL2 mRNA in five RCC cell lines (ACHN, Caki-1, RCZ, RCW, and OS-RC-2) has been analyzed by reverse transcriptase-polymerase chain reaction. The effects of siRNA containing human BCL2 sense and BCL2 antisense sequences (annealed and transfected with lipid) on the proliferation and viability of cultures of established human RCC cell lines can be determined by MTS assay. The expression of BCL2 protein in ACHN tumor cells following siRNA treatment can be evaluated by Western blot analysis, and the extent of apoptosis in these cells can be determined by fluorescence-activated cell sorter (FACS) analysis. The antitumor activity in ACHN xenografts in nu/nu mice is monitored by measuring differences in tumor weight in treated and control mice.

Animal Model Development

Tumor cell lines (ACHN, Caki-1, RCZ, RCW, and OS-RC-2) are characterized to establish their growth curves in mice. These cell lines are implanted into both nude and SCID mice and primary tumor volumes are measured three times per week. Growth

characteristics of these tumor lines using a Matrigel implantation format can also be established. The use of other cell lines that have been engineered to express high levels of BCL2 can also be used in the described studies. The tumor cell line(s) and implantation method that supports the most consistent and reliable tumor growth is used in animal studies testing the lead BCL2 nucleic acid(s). Nucleic acids are administered by daily subcutaneous injection or by continuous subcutaneous infusion from Alzet mini osmotic pumps beginning three days after tumor implantation and continuing for the duration of the study. Group sizes of at least 10 animals are employed. Efficacy is determined by statistical comparison of tumor volume of nucleic acid-treated animals to a control group of animals treated with saline alone. Because the growth of these tumors is generally slow (45-60 days), an initial endpoint is the time in days it takes to establish an easily measurable primary tumor (i.e. 50-100 mm^3) in the presence or absence of nucleic acid treatment.

BCL2 Protein Levels for Patient Screening and as a Potential Endpoint

Because elevated BCL2 levels can be detected in several cancers, cancer patients can be pre-screened for elevated BCL2 prior to admission to initial clinical trials testing an anti-BCL2 nucleic acid. Initial BCL2 levels can be determined (by ELISA) from tumor biopsies or resected tumor samples. During clinical trials, it may be possible to monitor circulating BCL2 protein by ELISA. Evaluation of serial blood/serum samples over the course of the anti-BCL2 nucleic acid treatment period could be useful in determining early indications of efficacy.

Example 9: RNAi mediated inhibition of BCL2 RNA expression

siNA constructs (**Table III**) are tested for efficacy in reducing BCL2 RNA expression in, for example, A549 cells. Cells are plated approximately 24h before transfection in 96-well plates at 5,000-7,500 cells/well, 100 µl/well, such that at the time of transfection cells are 70-90% confluent. For transfection, annealed siNAs are mixed with the transfection reagent (Lipofectamine 2000, Invitrogen) in a volume of 50 µl/well and incubated for 20 min. at room temperature. The siNA transfection mixtures are added to cells to give a final siNA concentration of 25 nM in a volume of 150 µl. Each siNA transfection mixture is added to 3 wells for triplicate siNA treatments. Cells are incubated at 37° for 24h in the continued presence of the siNA transfection mixture. At

24h, RNA is prepared from each well of treated cells. The supernatants with the transfection mixtures are first removed and discarded, then the cells are lysed and RNA prepared from each well. Target gene expression following treatment is evaluated by RT-PCR for the target gene and for a control gene (36B4, an RNA polymerase subunit) for normalization. The triplicate data is averaged and the standard deviations determined for each treatment. Normalized data are graphed and the percent reduction of target mRNA by active siNAs in comparison to their respective inverted control siNAs is determined.

In a non-limiting example, A549 cells were transfected with 0.25 ug/well of lipid complexed with 25 nM siNA. A siNA construct comprising ribonucleotides and 3'-terminal dithymidine caps (RPI#30998/31074) was tested along with a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro pyrimidine nucleotides and purine ribonucleotides in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxyabasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage (RPI#31368/31369), which was also compared to a matched chemistry inverted control (RPI#31370/31371) and a chemically modified siNA construct comprising 2'-deoxy-2'-fluoro pyrimidine and 2'-deoxy-2'-fluoro purine nucleotides in which the sense strand of the siNA is further modified with 5' and 3'-terminal inverted deoxyabasic caps and the antisense strand comprises a 3'-terminal phosphorothioate internucleotide linkage (RPI#31372/31373) which was also compared to a matched chemistry inverted control (RPI#31374/31375). In addition, the siNA constructs were also compared to untreated cells, cells transfected with lipid and scrambled siNA constructs (Scram1 and Scram2), and cells transfected with lipid alone (transfection control). As shown in Figure 12, the siNA constructs show significant reduction of BCL2 RNA expression compared to scrambled, untreated, and transfection controls. Additional stabilization chemistries as described in Table IV are similarly assayed for activity.

Example 10: Indications

Particular degenerative and disease states that can be associated with BCL2 expression modulation include, but are not limited to, cancer, including malignant blood diseases such as lymphomas (eg. non-Hodgkins and Hodgkins lymphomas), leukemias

(eg. chronic myeloid leukemia, CML; acute myeloid leukemias, AML; secondary leukemias, acute lymphoblastic leukemias, ALL; chronic lymphoid leukemia; CLL), polycytemia vera, idiopathic myelofibrosis, essential thrombocythemia, myelodysplastic syndromes, autoimmune disease (eg. multiple sclerosis, lupus, rheumatoid arthritis, insulin dependent diabetes, encephalitis, Rasmussen's encephalitis, thyroiditis, Crohn's disease, fibromyalgia, Grave's disease, Guillain Barre syndrome, chronic fatigue syndrome, autoimmune hepatitis, Meniere's disease, Myasthenia Gravis, cardiomyopathy, polymyalgia, Psoriasis, ulcerative collitis, etc.), viral infection (eg. HIV, HCV, HBV, RSV, CMV, HSV, influenza, rhinovirus etc.) and any other diseases or conditions that are related to the levels of BCL2 in a cell or tissue, alone or in combination with other therapies. The reduction of BCL2 expression (e.g., BCL2 RNA levels) and thus reduction in the level of the respective protein relieves, to some extent, the symptoms of the disease or condition.

The use of radiation treatments and chemotherapeutics such as Gemcytabine and cyclophosphamide are non-limiting examples of chemotherapeutic agents that can be combined with or used in conjunction with the nucleic acid molecules (*e.g.* siNA molecules) of the instant invention. Those skilled in the art will recognize that other anti-cancer compounds and therapies can similarly be readily combined with the nucleic acid molecules of the instant invention (*e.g.* siNA molecules) and are hence within the scope of the instant invention. Such compounds and therapies are well known in the art (see for example *Cancer: Principles and Prancticе of Oncology*, Volumes 1 and 2, eds Devita, V.T., Hellman, S., and Rosenberg, S.A., J.B. Lippincott Company, Philadelphia, USA; incorporated herein by reference) and include, without limitation, folates, antifolates, pyrimidine analogs, fluoropyrimidines, purine analogs, adenosine analogs, topoisomerase I inhibitors, anthrapyrazoles, retinoids, antibiotics, anthaBCL2s, platinum analogs, alkylating agents, nitrosoureas, plant derived compounds such as vinca alkaloids, epipodophyllotoxins, tyrosine kinase inhibitors, taxols, radiation therapy, surgery, nutritional supplements, gene therapy, radiotherapy, for example 3D-CRT, immunotoxin therapy, for example ricin, and monoclonal antibodies. Specific examples of chemotherapeutic compounds that can be combined with or used in conjuction with the nucleic acid molecules of the invention include, but are not limited to, Paclitaxel; Docetaxel; Methotrexate; Doxorubin; Edatrexate; Vinorelbine; Tomaxifen; Leucovorin; 5-fluoro uridine (5-FU); Ionotecan; Cisplatin; Carboplatin; Amsacrine; Cytarabine;

Bleomycin; Mitomycin C; Dactinomycin; Mithramycin; Hexamethylmelamine; Dacarbazine; L-asperginase; Nitrogen mustard; Melphalan, Chlorambucil; Busulfan; Ifosfamide; 4-hydroperoxycyclophosphamide, Thiotepa; Irinotecan (CAMPTOSAR®, CPT-11, Camptothecin-11, Campto) Tamoxifen, Herceptin; IMC C225; ABX-EGF: and combinations thereof. The above list provides non-limiting examples of compounds and/or methods that can be combined with or used in conjunction with the nucleic acid molecules (*e.g.* siNA) of the instant invention. Those skilled in the art will recognize that other drug compounds and therapies can similarly be readily combined with the nucleic acid molecules of the instant invention (e.g., siNA molecules) are hence within the scope of the instant invention.

Example 11: Diagnostic uses

The siNA molecules of the invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (e.g., RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of endogenous or exogenous, for example viral, RNA in a cell. The close relationship between siNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siNA molecules described in this invention, one can map nucleotide changes, which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with siNA molecules can be used to inhibit gene expression and define the role of specified gene products in the progression of disease or infection. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes, siNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siNA molecules and/or other chemical or biological molecules). Other *in vitro* uses of siNA molecules of this invention are well known in the art, and include detection of the

presence of mRNAs associated with a disease, infection, or related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a siNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

In a specific example, siNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (*i.e.*, those that cleave only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (*i.e.*, those that cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis requires two siNA molecules, two substrates and one unknown sample, which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, disease related or infection related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as

those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Table I: BCL2 Accession Numbers

LOCUS	BCL2	6030 bp mRNA linear PRI 03-FEB-2001
DEFINITION	Homo sapiens B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein, transcript variant alpha, mRNA.	
ACCESSION	NM_000633	
LOCUS	BCL2	911 bp mRNA linear PRI 03-FEB-2001
DEFINITION	Homo sapiens B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein, transcript variant beta, mRNA.	
ACCESSION	NM_000657	
LOCUS	AF401211	137 bp mRNA linear PRI 13-SEP-2001
DEFINITION	Homo sapiens BCL2 protein mRNA, partial cds.	
ACCESSION	AF401211	
LOCUS	BC027258	2704 bp mRNA linear PRI 08-APR-2002
DEFINITION	Homo sapiens, B-cell CLL/lymphoma 2, clone MGC:21366 IMAGE:4511027, mRNA, complete cds.	
ACCESSION	BC027258	
LOCUS	HUMBCL2A	5086 bp mRNA linear PRI 31-OCT-1994
DEFINITION	Human B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene mRNA encoding bcl-2-alpha protein, complete cds.	
ACCESSION	M13994	
LOCUS	HUMBCL2B	911 bp mRNA linear PRI 31-OCT-1994
DEFINITION	Human B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene mRNA encoding bcl-2-beta protein, complete cds.	
ACCESSION	M13995	

LOCUS HUMBCL2C 6030 bp mRNA linear PRI 27-APR-1993
DEFINITION Human bcl-2 mRNA.
ACCESSION M14745

LOCUS HSBCL2IG 1846 bp mRNA linear PRI 26-MAR-1993
DEFINITION H.sapiens mRNA for bcl2-Ig fusion gene.
ACCESSION X06487

LOCUS BCL2L1 2575 bp mRNA linear PRI 15-JAN-2003
DEFINITION Homo sapiens BCL2-like 1 (BCL2L1), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.
ACCESSION NM_138578

LOCUS BCL2L1 2386 bp mRNA linear PRI 15-JAN-2003
DEFINITION Homo sapiens BCL2-like 1 (BCL2L1), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.
ACCESSION NM_001191

LOCUS AF203373 816 bp mRNA linear PRI 20-AUG-2000
DEFINITION Homo sapiens myeloid cell leukemia-1 short protein (MCL1) mRNA, complete cds.
ACCESSION AF203373

LOCUS BCL2L11 223 bp mRNA linear PRI 05-NOV-2002
DEFINITION Homo sapiens BCL2-like 11 (apoptosis facilitator) (BCL2L11), transcript variant 8, mRNA.
ACCESSION NM_138627

LOCUS ced-9Co 843 bp mRNA linear INV 22-NOV-2002
DEFINITION Caenorhabditis elegans essential CYTochrome CYT-1, CEll Death

abnormality CED-9, abnormal MEthyl Viologen sensitivity MEV-1 (31.8 kD) (ced-9Co), alternative variant b, mRNA.
ACCESSION NM_066883

LOCUS BAG1 1311 bp mRNA linear PRI 01-NOV-2000
DEFINITION Homo sapiens BCL2-associated athanogene (BAG1), mRNA.
ACCESSION NM_004323

LOCUS AK094541 3107 bp mRNA linear PRI 15-JUL-2002
DEFINITION Homo sapiens cDNA FLJ37222 fis, clone BRAMY1000130, highly similar to Homo sapiens MAGE-E1b mRNA.
ACCESSION AK094541

LOCUS BC016281 829 bp mRNA linear PRI 05-NOV-2001
DEFINITION Homo sapiens, BCL2-related protein A1, clone MGC:8991 IMAGE:3920808, mRNA, complete cds.
ACCESSION BC016281

Table II: BCL2 siNA and Target Sequences

BCL2 = NM_000633

Pos	Target Sequence	Seq ID	UPos	Upper seq	Seq ID	LPos	Lower seq	Seq ID
3	GCCCGCCCCUCCGCGCCGC	1	3	GCCCGCCCCUCCGCGCCGC	1	25	GCGGCGCGGAGGGGCGGGC	415
21	CCUGCCCGCCCGCCCGCCG	2	21	CCUGCCCGCCCGCCCGCCG	2	41	CGGCGGGCGGGCGGGCAGG	416
39	GCGCUCCCGCCCGCCGCUC	3	39	GCGCUCCCGCCCGCCGCUC	3	59	GAGCGGCGGGCGGGAGCGC	417
57	CUCCGUGGCCCCGCCGCGC	4	57	CUCCGUGGCCCCGCCGCGC	4	77	GCGCGGCGGGGCCACGGAG	418
75	CUGCCGCCGCCGCCGCUGC	5	75	CUGCCGCCGCCGCCGCUGC	5	95	GCAGCGGCGGCGGCGGCAG	419
93	CCAGCGAAGGUGCCGGGGC	6	93	CCAGCGAAGGUGCCGGGGC	6	113	GCCCCGGCACCUUCGCUGG	420
111	CUCCGGGCCCUCCCUGCCG	7	111	CUCCGGGCCCUCCCUGCCG	7	131	CGGCAGGGAGGGCCCGGAG	421
129	GGCGGCCGUCAGCGCUCGG	8	129	GGCGGCCGUCAGCGCUCGG	8	149	CCGAGCGCUGACGGCCGCC	422
147	GAGCGAACUGCGCGACGGG	9	147	GAGCGAACUGCGCGACGGG	9	167	CCCGUCGCGCAGUUCGCUC	423
165	GAGGUCCGGGAGGCGACCG	10	165	GAGGUCCGGGAGGCGACCG	10	185	CGGUCGCCUCCCGGACCUC	424
183	GUAGUCGCGCCGCCGCGCA	11	183	GUAGUCGCGCCGCCGCGCA	11	203	UGCGCGGCGGCGCGACUAC	425
201	AGGACCAGGAGGAGGAGAA	12	201	AGGACCAGGAGGAGGAGAA	12	221	UUCUCCUCCUCCUGGUCCU	426
219	AAGGGUGCGCAGCCCGGAG	13	219	AAGGGUGCGCAGCCCGGAG	13	239	CUCCGGGCUGCGCACCCUU	427
237	GGCGGGGUGCGCCGGUGGG	14	237	GGCGGGGUGCGCCGGUGGG	14	257	CCCACCGGCGCACCCCGCC	428
255	GGUGCAGCGGAAGAGGGGG	15	255	GGUGCAGCGGAAGAGGGGG	15	275	CCCCCUCUUCCGCUGCACC	429
273	GUCCAGGGGGGAGAACUUC	16	273	GUCCAGGGGGGAGAACUUC	16	293	GAAGUUCUCCCCCCUGGAC	430
291	CGUAGCAGUCAUCCUUUUU	17	291	CGUAGCAGUCAUCCUUUUU	17	311	AAAAAGGAUGACUGCUACG	431
309	UAGGAAAAGAGGGAAAAAA	18	309	UAGGAAAAGAGGGAAAAAA	18	329	UUUUUUCCCUCUUUUCCUA	432
327	AUAAAACCCUCCCCCACCA	19	327	AUAAAACCCUCCCCCACCA	19	347	UGGUGGGGGAGGGUUUUAU	433
345	ACCUCCUUCUCCCCACCCC	20	345	ACCUCCUUCUCCCCACCCC	20	365	GGGGUGGGGAGAAGGAGGU	434
363	CUCGCCGCACCACACACAG	21	363	CUCGCCGCACCACACACAG	21	383	CUGUGUGUGGUGCGGCGAG	435
381	GCGCGGGCUUCUAGCGCUC	22	381	GCGCGGGCUUCUAGCGCUC	22	401	GAGCGCUAGAAGCCCGCGC	436
399	CGGCACCGGCGGGCCAGGC	23	399	CGGCACCGGCGGGCCAGGC	23	419	GCCUGGCCCGCCGGUGCCG	437
417	CGCGUCCUGCCUUCAUUUA	24	417	CGCGUCCUGCCUUCAUUUA	24	437	UAAAUGAAGGCAGGACGCG	438
435	AUCCAGCAGCUUUUCGGAA	25	435	AUCCAGCAGCUUUUCGGAA	25	455	UUCCGAAAAGCUGCUGGAU	439
453	AAAUGCAUUUGCUGUUCGG	26	453	AAAUGCAUUUGCUGUUCGG	26	473	CCGAACAGCAAAUGCAUUU	440
471	GAGUUUAAUCAGAAGACGA	27	471	GAGUUUAAUCAGAAGACGA	27	491	UCGUCUUCUGAUUAAACUC	441
489	AUUCCUGCCUCCGUCCCCG	28	489	AUUCCUGCCUCCGUCCCCG	28	509	CGGGGACGGAGGCAGGAAU	442
507	GGCUCCUUCAUCGUCCCAU	29	507	GGCUCCUUCAUCGUCCCAU	29	527	AUGGGACGAUGAAGGAGCC	443

525	UCUCCCCUGUCUCUCUCCU	30	525	UCUCCCCUGUCUCUCUCCU	30	545	AGGAGAGAGACAGGGGAGA	444
543	UGGGGAGGCGUGAAGCGGU	31	543	UGGGGAGGCGUGAAGCGGU	31	563	ACCGCUUCACGCCUCCCCA	445
561	UCCCGUGGAUAGAGAUUCA	32	561	UCCCGUGGAUAGAGAUUCA	32	581	UGAAUCUCUAUCCACGGGA	446
579	AUGCCUGUGUCCGCGCGUG	33	579	AUGCCUGUGUCCGCGCGUG	33	599	CACGCGCGGACACAGGCAU	447
597	GUGUGCGCGCGUAUAAAUU	34	597	GUGUGCGCGCGUAUAAAUU	34	617	AAUUUAUACGCGCGCACAC	448
615	UGCCGAGAAGGGGAAAACA	35	615	UGCCGAGAAGGGGAAAACA	35	635	UGUUUUCCCCUUCUCGGCA	449
633	AUCACAGGACUUCUGCGAA	36	633	AUCACAGGACUUCUGCGAA	36	653	UUCGCAGAAGUCCUGUGAU	450
651	AUACCGGACUGAAAAUUGU	37	651	AUACCGGACUGAAAAUUGU	37	671	ACAAUUUUCAGUCCGGUAU	451
669	UAAUUCAUCUGCCGCCGCC	38	669	UAAUUCAUCUGCCGCCGCC	38	689	GGCGGCGGCAGAUGAAUUA	452
687	CGCUGCCAAAAAAAAACUC	39	687	CGCUGCCAAAAAAAAACUC	39	707	GAGUUUUUUUUUGGCAGCG	453
705	CGAGCUCUUGAGAUCUCCG	40	705	CGAGCUCUUGAGAUCUCCG	40	725	CGGAGAUCUCAAGAGCUCG	454
723	GGUUGGGAUUCCUGCGGAU	41	723	GGUUGGGAUUCCUGCGGAU	41	743	AUCCGCAGGAAUCCCAACC	455
741	UUGACAUUUCUGUGAAGCA	42	741	UUGACAUUUCUGUGAAGCA	42	761	UGCUUCACAGAAAUGUCAA	456
759	AGAAGUCUGGGAAUCGAUC	43	759	AGAAGUCUGGGAAUCGAUC	43	779	GAUCGAUUCCCAGACUUCU	457
777	CUGGAAAUCCUCCUAAUUU	44	777	CUGGAAAUCCUCCUAAUUU	44	797	AAAUUAGGAGGAUUUCCAG	458
795	UUUACUCCCUCUCCCCCCG	45	795	UUUACUCCCUCUCCCCCCG	45	815	CGGGGGGAGAGGGAGUAAA	459
813	GACUCCUGAUUCAUUGGGA	46	813	GACUCCUGAUUCAUUGGGA	46	833	UCCCAAUGAAUCAGGAGUC	460
831	AAGUUUCAAAUCAGCUAUA	47	831	AAGUUUCAAAUCAGCUAUA	47	851	UAUAGCUGAUUUGAAACUU	461
849	AACUGGAGAGUGCUGAAGA	48	849	AACUGGAGAGUGCUGAAGA	48	869	UCUUCAGCACUCUCCAGUU	462
867	AUUGAUGGGAUCGUUGCCU	49	867	AUUGAUGGGAUCGUUGCCU	49	887	AGGCAACGAUCCCAUCAAU	463
885	UUAUGCAUUUGUUUUGGUU	50	885	UUAUGCAUUUGUUUUGGUU	50	905	AACCAAAACAAAUGCAUAA	464
903	UUUACAAAAAGGAAACUUG	51	903	UUUACAAAAAGGAAACUUG	51	923	CAAGUUUCCUUUUUGUAAA	465
921	GACAGAGGAUCAUGCUGUA	52	921	GACAGAGGAUCAUGCUGUA	52	941	UACAGCAUGAUCCUCUGUC	466
939	ACUUAAAAAAAUACAAGUAA	53	939	ACUUAAAAAAAUACAAGUAA	53	959	UUACUUGUAUUUUUUAAGU	467
957	AGUCUCGCACAGGAAAUUG	54	957	AGUCUCGCACAGGAAAUUG	54	977	CAAUUUCCUGUGCGAGACU	468
975	GGUUUAAUGUAACUUUCAA	55	975	GGUUUAAUGUAACUUUCAA	55	995	UUGAAAGUUACAUUAAACC	469
993	AUGGAAACCUUUGAGAUUU	56	993	AUGGAAACCUUUGAGAUUU	56	1013	AAAUCUCAAAGGUUUCCAU	470
1011	UUUUACUUAAAGUGCAUUC	57	1011	UUUUACUUAAAGUGCAUUC	57	1031	GAAUGCACUUUAAGUAAAA	471
1029	CGAGUAAAUUUAAUUUCCA	58	1029	CGAGUAAAUUUAAUUUCCA	58	1049	UGGAAAUUAAAUUUACUCG	472
1047	AGGCAGCUUAAUACAUUGU	59	1047	AGGCAGCUUAAUACAUUGU	59	1067	ACAAUGUAUUAAGCUGCCU	473
1065	UUUUUAGCCGUGUUACUUG	60	1065	UUUUUAGCCGUGUUACUUG	60	1085	CAAGUAACACGGCUAAAAA	474
1083	GUAGUGUGUAUGCCCUGCU	61	1083	GUAGUGUGUAUGCCCUGCU	61	1103	AGCAGGGCAUACACACUAC	475
1101	UUUCACUCAGUGUGUACAG	62	1101	UUUCACUCAGUGUGUACAG	62	1121	CUGUACACACUGAGUGAAA	476
1119	GGGAAACGCACCUGAUUUU	63	1119	GGGAAACGCACCUGAUUUU	63	1139	AAAAUCAGGUGCGUUUCCC	477
1137	UUUACUUAUUAGUUUGUUU	64	1137	UUUACUUAUUAGUUUGUUU	64	1157	AAACAAACUAAUAAGUAAA	478
1155	UUUUCUUUAACCUUUCAGC	65	1155	UUUUCUUUAACCUUUCAGC	65	1175	GCUGAAAGGUUAAAGAAAA	479

1173	CAUCACAGAGGAAGUAGAC	66	1173	CAUCACAGAGGAAGUAGAC	66	1193	GUCUACUUCCUCUGUGAUG	480
1191	CUGAUAUUAACAAUACUUA	67	1191	CUGAUAUUAACAAUACUUA	67	1211	UAAGUAUUGUUAAUAUCAG	481
1209	ACUAAUAAUAACGUGCCUC	68	1209	ACUAAUAAUAACGUGCCUC	68	1229	GAGGCACGUUAUUAUUAGU	482
1227	CAUGAAAUAAAGAUCCGAA	69	1227	CAUGAAAUAAAGAUCCGAA	69	1247	UUCGGAUCUUUAUUUCAUG	483
1245	AAGGAAUUGGAAUAAAAAU	70	1245	AAGGAAUUGGAAUAAAAAU	70	1265	AUUUUUAUUCCAAUUCCUU	484
1263	UUUCCUGCGUCUCAUGCCA	71	1263	UUUCCUGCGUCUCAUGCCA	71	1283	UGGCAUGAGACGCAGGAAA	485
1281	AAGAGGGAAACACCAGAAU	72	1281	AAGAGGGAAACACCAGAAU	72	1301	AUUCUGGUGUUUCCCUCUU	486
1299	UCAAGUGUUCCGCGUGAUU	73	1299	UCAAGUGUUCCGCGUGAUU	73	1319	AAUCACGCGGAACACUUGA	487
1317	UGAAGACACCCCCUCGUCC	74	1317	UGAAGACACCCCCUCGUCC	74	1337	GGACGAGGGGGUGUCUUCA	488
1335	CAAGAAUGCAAAGCACAUC	75	1335	CAAGAAUGCAAAGCACAUC	75	1355	GAUGUGCUUUGCAUUCUUG	489
1353	CCAAUAAAAUAGCUGGAUU	76	1353	CCAAUAAAAUAGCUGGAUU	76	1373	AAUCCAGCUAUUUUAUUGG	490
1371	UAUAACUCCUCUUCUUUCU	77	1371	UAUAACUCCUCUUCUUUCU	77	1391	AGAAAGAAGAGGAGUUAUA	491
1389	UCUGGGGGCCGUGGGGUGG	78	1389	UCUGGGGGCCGUGGGGUGG	78	1409	CCACCCCACGGCCCCCAGA	492
1407	GGAGCUGGGGCGAGAGGUG	79	1407	GGAGCUGGGGCGAGAGGUG	79	1427	CACCUCUCGCCCCAGCUCC	493
1425	GCCGUUGGCCCCCGUUGCU	80	1425	GCCGUUGGCCCCCGUUGCU	80	1445	AGCAACGGGGGCCAACGGC	494
1443	UUUUCCUCUGGGAAGGAUG	81	1443	UUUUCCUCUGGGAAGGAUG	81	1463	CAUCCUUCCCAGAGGAAAA	495
1461	GGCGCACGCUGGGAGAACG	82	1461	GGCGCACGCUGGGAGAACG	82	1481	CGUUCUCCCAGCGUGCGCC	496
1479	GGGGUACGACAACCGGGAG	83	1479	GGGGUACGACAACCGGGAG	83	1499	CUCCCGGUUGUCGUACCCC	497
1497	GAUAGUGAUGAAGUACAUC	84	1497	GAUAGUGAUGAAGUACAUC	84	1517	GAUGUACUUCAUCACUAUC	498
1515	CCAUUAUAAGCUGUCGCAG	85	1515	CCAUUAUAAGCUGUCGCAG	85	1535	CUGCGACAGCUUAUAAUGG	499
1533	GAGGGGCUACGAGUGGGAU	86	1533	GAGGGGCUACGAGUGGGAU	86	1553	AUCCCACUCGUAGCCCCUC	500
1551	UGCGGGAGAUGUGGGCGCC	87	1551	UGCGGGAGAUGUGGGCGCC	87	1571	GGCGCCCACAUCUCCCGCA	501
1569	CGCGCCCCCGGGGGCCGCC	88	1569	CGCGCCCCCGGGGGCCGCC	88	1589	GGCGGCCCCCGGGGGCGCG	502
1587	CCCCGCACCGGGCAUCUUC	89	1587	CCCCGCACCGGGCAUCUUC	89	1607	GAAGAUGCCCGGUGCGGGG	503
1605	CUCCUCCCAGCCCGGGCAC	90	1605	CUCCUCCCAGCCCGGGCAC	90	1625	GUGCCCGGGCUGGGAGGAG	504
1623	CACGCCCCAUCCAGCCGCA	91	1623	CACGCCCCAUCCAGCCGCA	91	1643	UGCGGCUGGAUGGGGCGUG	505
1641	AUCCCGCGACCCGGUCGCC	92	1641	AUCCCGCGACCCGGUCGCC	92	1661	GGCGACCGGGUCGCGGGAU	506
1659	CAGGACCUCGCCGCUGCAG	93	1659	CAGGACCUCGCCGCUGCAG	93	1679	CUGCAGCGGCGAGGUCCUG	507
1677	GACCCCGGCUGCCCCCGGC	94	1677	GACCCCGGCUGCCCCCGGC	94	1697	GCCGGGGGCAGCCGGGGUC	508
1695	CGCCGCCGCGGGGCCUGCG	95	1695	CGCCGCCGCGGGGCCUGCG	95	1715	CGCAGGCCCCGCGGCGGCG	509
1713	GCUCAGCCCGGUGCCACCU	96	1713	GCUCAGCCCGGUGCCACCU	96	1733	AGGUGGCACCGGGCUGAGC	510
1731	UGUGGUCCACCUGGCCCUC	97	1731	UGUGGUCCACCUGGCCCUC	97	1751	GAGGGCCAGGUGGACCACA	511
1749	CCGCCAAGCCGGCGACGAC	98	1749	CCGCCAAGCCGGCGACGAC	98	1769	GUCGUCGCCGGCUUGGCGG	512
1767	CUUCUCCCGCCGCUACCGC	99	1767	CUUCUCCCGCCGCUACCGC	99	1787	GCGGUAGCGGCGGGAGAAG	513
1785	CGGCGACUUCGCCGAGAUG	100	1785	CGGCGACUUCGCCGAGAUG	100	1805	CAUCUCGGCGAAGUCGCCG	514
1803	GUCCAGCCAGCUGCACCUG	101	1803	GUCCAGCCAGCUGCACCUG	101	1823	CAGGUGCAGCUGGCUGGAC	515

1821	GACGCCCUUCACCGCGCGG	102	1821	GACGCCCUUCACCGCGCGG	102	1841	CCGCGCGGUGAAGGGCGUC	516
1839	GGGACGCUUUGCCACGGUG	103	1839	GGGACGCUUUGCCACGGUG	103	1859	CACCGUGGCAAAGCGUCCC	517
1857	GGUGGAGGAGCUCUUCAGG	104	1857	GGUGGAGGAGCUCUUCAGG	104	1877	CCUGAAGAGCUCCUCCACC	518
1875	GGACGGGGUGAACUGGGGG	105	1875	GGACGGGGUGAACUGGGGG	105	1895	CCCCCAGUUCACCCCGUCC	519
1893	GAGGAUUGUGGCCUUCUUU	106	1893	GAGGAUUGUGGCCUUCUUU	106	1913	AAAGAAGGCCACAAUCCUC	520
1911	UGAGUUCGGUGGGGUCAUG	107	1911	UGAGUUCGGUGGGGUCAUG	107	1931	CAUGACCCCACCGAACUCA	521
1929	GUGUGUGGAGAGCGUCAAC	108	1929	GUGUGUGGAGAGCGUCAAC	108	1949	GUUGACGCUCUCCACACAC	522
1947	CCGGGAGAUGUCGCCCCUG	109	1947	CCGGGAGAUGUCGCCCCUG	109	1967	CAGGGGCGACAUCUCCCGG	523
1965	GGUGGACAACAUCGCCCUG	110	1965	GGUGGACAACAUCGCCCUG	110	1985	CAGGGCGAUGUUGUCCACC	524
1983	GUGGAUGACUGAGUACCUG	111	1983	GUGGAUGACUGAGUACCUG	111	2003	CAGGUACUCAGUCAUCCAC	525
2001	GAACCGGCACCUGCACACC	112	2001	GAACCGGCACCUGCACACC	112	2021	GGUGUGCAGGUGCCGGUUC	526
2019	CUGGAUCCAGGAUAACGGA	113	2019	CUGGAUCCAGGAUAACGGA	113	2039	UCCGUUAUCCUGGAUCCAG	527
2037	AGGCUGGGAUGCCUUUGUG	114	2037	AGGCUGGGAUGCCUUUGUG	114	2057	CACAAAGGCAUCCCAGCCU	528
2055	GGAACUGUACGGCCCCAGC	115	2055	GGAACUGUACGGCCCCAGC	115	2075	GCUGGGGCCGUACAGUUCC	529
2073	CAUGCGGCCUCUGUUUGAU	116	2073	CAUGCGGCCUCUGUUUGAU	116	2093	AUCAAACAGAGGCCGCAUG	530
2091	UUUCUCCUGGCUGUCUCUG	117	2091	UUUCUCCUGGCUGUCUCUG	117	2111	CAGAGACAGCCAGGAGAAA	531
2109	GAAGACUCUGCUCAGUUUG	118	2109	GAAGACUCUGCUCAGUUUG	118	2129	CAAACUGAGCAGAGUCUUC	532
2127	GGCCCUGGUGGGAGCUUGC	119	2127	GGCCCUGGUGGGAGCUUGC	119	2147	GCAAGCUCCCACCAGGGCC	533
2145	CAUCACCCUGGGUGCCUAU	120	2145	CAUCACCCUGGGUGCCUAU	120	2165	AUAGGCACCCAGGGUGAUG	534
2163	UCUGAGCCACAAGUGAAGU	121	2163	UCUGAGCCACAAGUGAAGU	121	2183	ACUUCACUUGUGGCUCAGA	535
2181	UCAACAUGCCUGCCCCAAA	122	2181	UCAACAUGCCUGCCCCAAA	122	2201	UUUGGGGCAGGCAUGUUGA	536
2199	ACAAAUAUGCAAAAGGUUC	123	2199	ACAAAUAUGCAAAAGGUUC	123	2219	GAACCUUUUGCAUAUUUGU	537
2217	CACUAAAGCAGUAGAAAUA	124	2217	CACUAAAGCAGUAGAAAUA	124	2237	UAUUUCUACUGCUUUAGUG	538
2235	AAUAUGCAUUGUCAGUGAU	125	2235	AAUAUGCAUUGUCAGUGAU	125	2255	AUCACUGACAAUGCAUAUU	539
2253	UGUACCAUGAAACAAAGCU	126	2253	UGUACCAUGAAACAAAGCU	126	2273	AGCUUUGUUUCAUGGUACA	540
2271	UGCAGGCUGUUUAAGAAAA	127	2271	UGCAGGCUGUUUAAGAAAA	127	2291	UUUUCUUAAACAGCCUGCA	541
2289	AAAUAACACACAUAUAAAC	128	2289	AAAUAACACACAUAUAAAC	128	2309	GUUUAUAUGUGUGUUAUUU	542
2307	CAUCACACACACAGACAGA	129	2307	CAUCACACACACAGACAGA	129	2327	UCUGUCUGUGUGUGUGAUG	543
2325	ACACACACACACACAACAA	130	2325	ACACACACACACACAACAA	130	2345	UUGUUGUGUGUGUGUGUGU	544
2343	AUUAACAGUCUUCAGGCAA	131	2343	AUUAACAGUCUUCAGGCAA	131	2363	UUGCCUGAAGACUGUUAAU	545
2361	AAACGUCGAAUCAGCUAUU	132	2361	AAACGUCGAAUCAGCUAUU	132	2381	AAUAGCUGAUUCGACGUUU	546
2379	UUACUGCCAAAGGGAAAUA	133	2379	UUACUGCCAAAGGGAAAUA	133	2399	UAUUUCCCUUUGGCAGUAA	547
2397	AUCAUUUAUUUUUUACAUU	134	2397	AUCAUUUAUUUUUUACAUU	134	2417	AAUGUAAAAAAUAAAUGAU	548
2415	UAUUAAGAAAAAAGAUUUA	135	2415	UAUUAAGAAAAAAGAUUUA	135	2435	UAAAUCUUUUUUCUUAAUA	549
2433	AUUUAUUUAAGACAGUCCC	136	2433	AUUUAUUUAAGACAGUCCC	136	2453	GGGACUGUCUUAAAUAAAU	550
2451	CAUCAAAACUCCGUCUUUG	137	2451	CAUCAAAACUCCGUCUUUG	137	2471	CAAAGACGGAGUUUUGAUG	551

2469	GGAAAUCCGACCACUAAUU	138	2469	GGAAAUCCGACCACUAAUU	138	2489	AAUUAGUGGUCGGAUUUCC	552
2487	UGCCAAACACCGCUUCGUG	139	2487	UGCCAAACACCGCUUCGUG	139	2507	CACGAAGCGGUGUUUGGCA	553
2505	GUGGCUCCACCUGGAUGUU	140	2505	GUGGCUCCACCUGGAUGUU	140	2525	AACAUCCAGGUGGAGCCAC	554
2523	UCUGUGCCUGUAAACAUAG	141	2523	UCUGUGCCUGUAAACAUAG	141	2543	CUAUGUUUACAGGCACAGA	555
2541	GAUUCGCUUUCCAUGUUGU	142	2541	GAUUCGCUUUCCAUGUUGU	142	2561	ACAACAUGGAAAGCGAAUC	556
2559	UUGGCCGGAUCACCAUCUG	143	2559	UUGGCCGGAUCACCAUCUG	143	2579	CAGAUGGUGAUCCGGCCAA	557
2577	GAAGAGCAGACGGAUGGAA	144	2577	GAAGAGCAGACGGAUGGAA	144	2597	UUCCAUCCGUCUGCUCUUC	558
2595	AAAAGGACCUGAUCAUUGG	145	2595	AAAAGGACCUGAUCAUUGG	145	2615	CCAAUGAUCAGGUCCUUUU	559
2613	GGGAAGCUGGCUUUCUGGC	146	2613	GGGAAGCUGGCUUUCUGGC	146	2633	GCCAGAAAGCCAGCUUCCC	560
2631	CUGCUGGAGGCUGGGGAGA	147	2631	CUGCUGGAGGCUGGGGAGA	147	2651	UCUCCCCAGCCUCCAGCAG	561
2649	AAGGUGUUCAUUCACUUGC	148	2649	AAGGUGUUCAUUCACUUGC	148	2669	GCAAGUGAAUGAACACCUU	562
2667	CAUUUCUUUGCCCUGGGGG	149	2667	CAUUUCUUUGCCCUGGGGG	149	2687	CCCCCAGGGCAAAGAAAUG	563
2685	GCGUGAUAUUAACAGAGGG	150	2685	GCGUGAUAUUAACAGAGGG	150	2705	CCCUCUGUUAAUAUCACGC	564
2703	GAGGGUUCCCGUGGGGGGA	151	2703	GAGGGUUCCCGUGGGGGGA	151	2723	UCCCCCCACGGGAACCCUC	565
2721	AAGUCCAUGCCUCCCUGGC	152	2721	AAGUCCAUGCCUCCCUGGC	152	2741	GCCAGGGAGGCAUGGACUU	566
2739	CCUGAAGAAGAGACUCUUU	153	2739	CCUGAAGAAGAGACUCUUU	153	2759	AAAGAGUCUCUUCUUCAGG	567
2757	UGCAUAUGACUCACAUGAU	154	2757	UGCAUAUGACUCACAUGAU	154	2777	AUCAUGUGAGUCAUAUGCA	568
2775	UGCAUACCUGGUGGGAGGA	155	2775	UGCAUACCUGGUGGGAGGA	155	2795	UCCUCCCACCAGGUAUGCA	569
2793	AAAAGAGUUGGGAACUUCA	156	2793	AAAAGAGUUGGGAACUUCA	156	2813	UGAAGUUCCCAACUCUUUU	570
2811	AGAUGGACCUAGUACCCAC	157	2811	AGAUGGACCUAGUACCCAC	157	2831	GUGGGUACUAGGUCCAUCU	571
2829	CUGAGAUUUCCACGCCGAA	158	2829	CUGAGAUUUCCACGCCGAA	158	2849	UUCGGCGUGGAAAUCUCAG	572
2847	AGGACAGCGAUGGGAAAAA	159	2847	AGGACAGCGAUGGGAAAAA	159	2867	UUUUUCCCAUCGCUGUCCU	573
2865	AUGCCCUUAAAUCAUAGGA	160	2865	AUGCCCUUAAAUCAUAGGA	160	2885	UCCUAUGAUUUAAGGGCAU	574
2883	AAAGUAUUUUUUUAAGCUA	161	2883	AAAGUAUUUUUUUAAGCUA	161	2903	UAGCUUAAAAAAAUACUUU	575
2901	ACCAAUUGUGCCGAGAAAA	162	2901	ACCAAUUGUGCCGAGAAAA	162	2921	UUUUCUCGGCACAAUUGGU	576
2919	AGCAUUUUAGCAAUUUAUA	163	2919	AGCAUUUUAGCAAUUUAUA	163	2939	UAUAAAUUGCUAAAAUGCU	577
2937	ACAAUAUCAUCCAGUACCU	164	2937	ACAAUAUCAUCCAGUACCU	164	2957	AGGUACUGGAUGAUAUUGU	578
2955	UUAAACCCUGAUUGUGUAU	165	2955	UUAAACCCUGAUUGUGUAU	165	2975	AUACACAAUCAGGGUUUAA	579
2973	UAUUCAUAUAUUUUGGAUA	166	2973	UAUUCAUAUAUUUUGGAUA	166	2993	UAUCCAAAAUAUAUGAAUA	580
2991	ACGCACCCCCCAACUCCCA	167	2991	ACGCACCCCCCAACUCCCA	167	3011	UGGGAGUUGGGGGGUGCGU	581
3009	AAUACUGGCUCUGUCUGAG	168	3009	AAUACUGGCUCUGUCUGAG	168	3029	CUCAGACAGAGCCAGUAUU	582
3027	GUAAGAAACAGAAUCCUCU	169	3027	GUAAGAAACAGAAUCCUCU	169	3047	AGAGGAUUCUGUUUCUUAC	583
3045	UGGAACUUGAGGAAGUGAA	170	3045	UGGAACUUGAGGAAGUGAA	170	3065	UUCACUUCCUCAAGUUCCA	584
3063	ACAUUUCGGUGACUUCCGA	171	3063	ACAUUUCGGUGACUUCCGA	171	3083	UCGGAAGUCACCGAAAUGU	585
3081	AUCAGGAAGGCUAGAGUUA	172	3081	AUCAGGAAGGCUAGAGUUA	172	3101	UAACUCUAGCCUUCCUGAU	586
3099	ACCCAGAGCAUCAGGCCGC	173	3099	ACCCAGAGCAUCAGGCCGC	173	3119	GCGGCCUGAUGCUCUGGGU	587

3117	CCACAAGUGCCUGCUUUUA	174	3117	CCACAAGUGCCUGCUUUUA	174	3137	UAAAAGCAGGCACUUGUGG	588
3135	AGGAGACCGAAGUCCGCAG	175	3135	AGGAGACCGAAGUCCGCAG	175	3155	CUGCGGACUUCGGUCUCCU	589
3153	GAACCUACCUGUGUCCCAG	176	3153	GAACCUACCUGUGUCCCAG	176	3173	CUGGGACACAGGUAGGUUC	590
3171	GCUUGGAGGCCUGGUCCUG	177	3171	GCUUGGAGGCCUGGUCCUG	177	3191	CAGGACCAGGCCUCCAAGC	591
3189	GGAACUGAGCCGGGCCCUC	178	3189	GGAACUGAGCCGGGCCCUC	178	3209	GAGGGCCCGGCUCAGUUCC	592
3207	CACUGGCCUCCUCCAGGGA	179	3207	CACUGGCCUCCUCCAGGGA	179	3227	UCCCUGGAGGAGGCCAGUG	593
3225	AUGAUCAACAGGGUAGUGU	180	3225	AUGAUCAACAGGGUAGUGU	180	3245	ACACUACCCUGUUGAUCAU	594
3243	UGGUCUCCGAAUGUCUGGA	181	3243	UGGUCUCCGAAUGUCUGGA	181	3263	UCCAGACAUUCGGAGACCA	595
3261	AAGCUGAUGGAUGGAGCUC	182	3261	AAGCUGAUGGAUGGAGCUC	182	3281	GAGCUCCAUCCAUCAGCUU	596
3279	CAGAAUUCCACUGUCAAGA	183	3279	CAGAAUUCCACUGUCAAGA	183	3299	UCUUGACAGUGGAAUUCUG	597
3297	AAAGAGCAGUAGAGGGGUG	184	3297	AAAGAGCAGUAGAGGGGUG	184	3317	CACCCCUCUACUGCUCUUU	598
3315	GUGGCUGGGCCUGUCACCC	185	3315	GUGGCUGGGCCUGUCACCC	185	3335	GGGUGACAGGCCCAGCCAC	599
3333	CUGGGGCCCUCCAGGUAGG	186	3333	CUGGGGCCCUCCAGGUAGG	186	3353	CCUACCUGGAGGGCCCCAG	600
3351	GCCCGUUUUCACGUGGAGC	187	3351	GCCCGUUUUCACGUGGAGC	187	3371	GCUCCACGUGAAAACGGGC	601
3369	CAUAGGAGCCACGACCCUU	188	3369	CAUAGGAGCCACGACCCUU	188	3389	AAGGGUCGUGGCUCCUAUG	602
3387	UCUUAAGACAUGUAUCACU	189	3387	UCUUAAGACAUGUAUCACU	189	3407	AGUGAUACAUGUCUUAAGA	603
3405	UGUAGAGGGAAGGAACAGA	190	3405	UGUAGAGGGAAGGAACAGA	190	3425	UCUGUUCCUUCCCUCUACA	604
3423	AGGCCCUGGGCCUUCCUAU	191	3423	AGGCCCUGGGCCUUCCUAU	191	3443	AUAGGAAGGCCCAGGGCCU	605
3441	UCAGAAGGACAUGGUGAAG	192	3441	UCAGAAGGACAUGGUGAAG	192	3461	CUUCACCAUGUCCUUCUGA	606
3459	GGCUGGGAACGUGAGGAGA	193	3459	GGCUGGGAACGUGAGGAGA	193	3479	UCUCCUCACGUUCCCAGCC	607
3477	AGGCAAUGGCCACGGCCCA	194	3477	AGGCAAUGGCCACGGCCCA	194	3497	UGGGCCGUGGCCAUUGCCU	608
3495	AUUUUGGCUGUAGCACAUG	195	3495	AUUUUGGCUGUAGCACAUG	195	3515	CAUGUGCUACAGCCAAAAU	609
3513	GGCACGUUGGCUGUGUGGC	196	3513	GGCACGUUGGCUGUGUGGC	196	3533	GCCACACAGCCAACGUGCC	610
3531	CCUUGGCCACCUGUGAGUU	197	3531	CCUUGGCCACCUGUGAGUU	197	3551	AACUCACAGGUGGCCAAGG	611
3549	UUAAAGCAAGGCUUUAAAU	198	3549	UUAAAGCAAGGCUUUAAAU	198	3569	AUUUAAAGCCUUGCUUUAA	612
3567	UGACUUUGGAGAGGGUCAC	199	3567	UGACUUUGGAGAGGGUCAC	199	3587	GUGACCCUCUCCAAAGUCA	613
3585	CAAAUCCUAAAAGAAGCAU	200	3585	CAAAUCCUAAAAGAAGCAU	200	3605	AUGCUUCUUUUAGGAUUUG	614
3603	UUGAAGUGAGGUGUCAUGG	201	3603	UUGAAGUGAGGUGUCAUGG	201	3623	CCAUGACACCUCACUUCAA	615
3621	GAUUAAUUGACCCCUGUCU	202	3621	GAUUAAUUGACCCCUGUCU	202	3641	AGACAGGGGUCAAUUAAUC	616
3639	UAUGGAAUUACAUGUAAAA	203	3639	UAUGGAAUUACAUGUAAAA	203	3659	UUUUACAUGUAAUUCCAUA	617
3657	ACAUUAUCUUGUCACUGUA	204	3657	ACAUUAUCUUGUCACUGUA	204	3677	UACAGUGACAAGAUAAUGU	618
3675	AGUUUGGUUUUAUUUGAAA	205	3675	AGUUUGGUUUUAUUUGAAA	205	3695	UUUCAAAUAAAACCAAACU	619
3693	AACCUGACAAAAAAAAAGU	206	3693	AACCUGACAAAAAAAAAGU	206	3713	ACUUUUUUUUUGUCAGGUU	620
3711	UUCCAGGUGUGGAAUAUGG	207	3711	UUCCAGGUGUGGAAUAUGG	207	3731	CCAUAUUCCACACCUGGAA	621
3729	GGGGUUAUCUGUACAUCCU	208	3729	GGGGUUAUCUGUACAUCCU	208	3749	AGGAUGUACAGAUAACCCC	622
3747	UGGGGCAUUAAAAAAAAAU	209	3747	UGGGGCAUUAAAAAAAAAU	209	3767	AUUUUUUUUUAAUGCCCCA	623

3765	UCAAUGGUGGGGAACUAUA	210	3765	UCAAUGGUGGGGAACUAUA	210	3785	UAUAGUUCCCCACCAUUGA	624
3783	AAAGAAGUAACAAAAGAAG	211	3783	AAAGAAGUAACAAAAGAAG	211	3803	CUUCUUUUGUUACUUCUUU	625
3801	GUGACAUCUUCAGCAAAUA	212	3801	GUGACAUCUUCAGCAAAUA	212	3821	UAUUUGCUGAAGAUGUCAC	626
3819	AAACUAGGAAAUUUUUUUU	213	3819	AAACUAGGAAAUUUUUUUU	213	3839	AAAAAAAAUUUCCUAGUUU	627
3837	UUCUUCCAGUUUAGAAUCA	214	3837	UUCUUCCAGUUUAGAAUCA	214	3857	UGAUUCUAAACUGGAAGAA	628
3855	AGCCUUGAAACAUUGAUGG	215	3855	AGCCUUGAAACAUUGAUGG	215	3875	CCAUCAAUGUUUCAAGGCU	629
3873	GAAUAACUCUGUGGCAUUA	216	3873	GAAUAACUCUGUGGCAUUA	216	3893	UAAUGCCACAGAGUUAUUC	630
3891	AUUGCAUUAUAUACCAUUU	217	3891	AUUGCAUUAUAUACCAUUU	217	3911	AAAUGGUAUAUAAUGCAAU	631
3909	UAUCUGUAUUAACUUUGGA	218	3909	UAUCUGUAUUAACUUUGGA	218	3929	UCCAAAGUUAAUACAGAUA	632
3927	AAUGUACUCUGUUCAAUGU	219	3927	AAUGUACUCUGUUCAAUGU	219	3947	ACAUUGAACAGAGUACAUU	633
3945	UUUAAUGCUGUGGUUGAUA	220	3945	UUUAAUGCUGUGGUUGAUA	220	3965	UAUCAACCACAGCAUUAAA	634
3963	AUUUCGAAAGCUGCUUUAA	221	3963	AUUUCGAAAGCUGCUUUAA	221	3983	UUAAAGCAGCUUUCGAAAU	635
3981	AAAAAAUACAUGCAUCUCA	222	3981	AAAAAAUACAUGCAUCUCA	222	4001	UGAGAUGCAUGUAUUUUUU	636
3999	AGCGUUUUUUUGUUUUUAA	223	3999	AGCGUUUUUUUGUUUUUAA	223	4019	UUAAAAACAAAAAAACGCU	637
4017	AUUGUAUUUAGUUAUGGCC	224	4017	AUUGUAUUUAGUUAUGGCC	224	4037	GGCCAUAACUAAAUACAAU	638
4035	CUAUACACUAUUUGUGAGC	225	4035	CUAUACACUAUUUGUGAGC	225	4055	GCUCACAAAUAGUGUAUAG	639
4053	CAAAGGUGAUCGUUUUCUG	226	4053	CAAAGGUGAUCGUUUUCUG	226	4073	CAGAAAACGAUCACCUUUG	640
4071	GUUUGAGAUUUUUAUCUCU	227	4071	GUUUGAGAUUUUUAUCUCU	227	4091	AGAGAUAAAAAUCUCAAAC	641
4089	UUGAUUCUUCAAAAGCAUU	228	4089	UUGAUUCUUCAAAAGCAUU	228	4109	AAUGCUUUUGAAGAAUCAA	642
4107	UCUGAGAAGGUGAGAUAAG	229	4107	UCUGAGAAGGUGAGAUAAG	229	4127	CUUAUCUCACCUUCUCAGA	643
4125	GCCCUGAGUCUCAGCUACC	230	4125	GCCCUGAGUCUCAGCUACC	230	4145	GGUAGCUGAGACUCAGGGC	644
4143	CUAAGAAAAACCUGGAUGU	231	4143	CUAAGAAAAACCUGGAUGU	231	4163	ACAUCCAGGUUUUUCUUAG	645
4161	UCACUGGCCACUGAGGAGC	232	4161	UCACUGGCCACUGAGGAGC	232	4181	GCUCCUCAGUGGCCAGUGA	646
4179	CUUUGUUUCAACCAAGUCA	233	4179	CUUUGUUUCAACCAAGUCA	233	4199	UGACUUGGUUGAAACAAAG	647
4197	AUGUGCAUUUCCACGUCAA	234	4197	AUGUGCAUUUCCACGUCAA	234	4217	UUGACGUGGAAAUGCACAU	648
4215	ACAGAAUUGUUUAUUGUGA	235	4215	ACAGAAUUGUUUAUUGUGA	235	4235	UCACAAUAAACAAUUCUGU	649
4233	ACAGUUAUAUCUGUUGUCC	236	4233	ACAGUUAUAUCUGUUGUCC	236	4253	GGACAACAGAUAUAACUGU	650
4251	CCUUUGACCUUGUUUCUUG	237	4251	CCUUUGACCUUGUUUCUUG	237	4271	CAAGAAACAAGGUCAAAGG	651
4269	GAAGGUUUCCUCGUCCCUG	238	4269	GAAGGUUUCCUCGUCCCUG	238	4289	CAGGGACGAGGAAACCUUC	652
4287	GGGCAAUUCCGCAUUUAAU	239	4287	GGGCAAUUCCGCAUUUAAU	239	4307	AUUAAAUGCGGAAUUGCCC	653
4305	UUCAUGGUAUUCAGGAUUA	240	4305	UUCAUGGUAUUCAGGAUUA	240	4325	UAAUCCUGAAUACCAUGAA	654
4323	ACAUGCAUGUUUGGUUAAA	241	4323	ACAUGCAUGUUUGGUUAAA	241	4343	UUUAACCAAACAUGCAUGU	655
4341	ACCCAUGAGAUUCAUUCAG	242	4341	ACCCAUGAGAUUCAUUCAG	242	4361	CUGAAUGAAUCUCAUGGGU	656
4359	GUUAAAAAUCCAGAUGGCG	243	4359	GUUAAAAAUCCAGAUGGCG	243	4379	CGCCAUCUGGAUUUUUAAC	657
4377	GAAUGACCAGCAGAUUCAA	244	4377	GAAUGACCAGCAGAUUCAA	244	4397	UUGAAUCUGCUGGUCAUUC	658
4395	AAUCUAUGGUGGUUUGACC	245	4395	AAUCUAUGGUGGUUUGACC	245	4415	GGUCAAACCACCAUAGAUU	659

4413	CUUUAGAGAGUUGCUUUAC	246	4413	CUUUAGAGAGUUGCUUUAC	246	4433	GUAAAGCAACUCUCUAAAG	660
4431	CGUGGCCUGUUUCAACACA	247	4431	CGUGGCCUGUUUCAACACA	247	4451	UGUGUUGAAACAGGCCACG	661
4449	AGACCCACCCAGAGCCCUC	248	4449	AGACCCACCCAGAGCCCUC	248	4469	GAGGGCUCUGGGUGGGUCU	662
4467	CCUGCCCUCCUUCCGCGGG	249	4467	CCUGCCCUCCUUCCGCGGG	249	4487	CCCGCGGAAGGAGGGCAGG	663
4485	GGGCUUUCUCAUGGCUGUC	250	4485	GGGCUUUCUCAUGGCUGUC	250	4505	GACAGCCAUGAGAAAGCCC	664
4503	CCUUCAGGGUCUUCCUGAA	251	4503	CCUUCAGGGUCUUCCUGAA	251	4523	UUCAGGAAGACCCUGAAGG	665
4521	AAUGCAGUGGUCGUUACGC	252	4521	AAUGCAGUGGUCGUUACGC	252	4541	GCGUAACGACCACUGCAUU	666
4539	CUCCACCAAGAAAGCAGGA	253	4539	CUCCACCAAGAAAGCAGGA	253	4559	UCCUGCUUUCUUGGUGGAG	667
4557	AAACCUGUGGUAUGAAGCC	254	4557	AAACCUGUGGUAUGAAGCC	254	4577	GGCUUCAUACCACAGGUUU	668
4575	CAGACCUCCCCGGCGGGCC	255	4575	CAGACCUCCCCGGCGGGCC	255	4595	GGCCCGCCGGGGAGGUCUG	669
4593	CUCAGGGAACAGAAUGAUC	256	4593	CUCAGGGAACAGAAUGAUC	256	4613	GAUCAUUCUGUUCCCUGAG	670
4611	CAGACCUUUGAAUGAUUCU	257	4611	CAGACCUUUGAAUGAUUCU	257	4631	AGAAUCAUUCAAAGGUCUG	671
4629	UAAUUUUUAAGCAAAAUAU	258	4629	UAAUUUUUAAGCAAAAUAU	258	4649	AUAUUUUGCUUAAAAAUUA	672
4647	UUAUUUUAUGAAAGGUUUA	259	4647	UUAUUUUAUGAAAGGUUUA	259	4667	UAAACCUUUCAUAAAAUAA	673
4665	ACAUUGUCAAAGUGAUGAA	260	4665	ACAUUGUCAAAGUGAUGAA	260	4685	UUCAUCACUUUGACAAUGU	674
4683	AUAUGGAAUAUCCAAUCCU	261	4683	AUAUGGAAUAUCCAAUCCU	261	4703	AGGAUUGGAUAUUCCAUAU	675
4701	UGUGCUGCUAUCCUGCCAA	262	4701	UGUGCUGCUAUCCUGCCAA	262	4721	UUGGCAGGAUAGCAGCACA	676
4719	AAAUCAUUUUAAUGGAGUC	263	4719	AAAUCAUUUUAAUGGAGUC	263	4739	GACUCCAUUAAAAUGAUUU	677
4737	CAGUUUGCAGUAUGCUCCA	264	4737	CAGUUUGCAGUAUGCUCCA	264	4757	UGGAGCAUACUGCAAACUG	678
4755	ACGUGGUAAGAUCCUCCAA	265	4755	ACGUGGUAAGAUCCUCCAA	265	4775	UUGGAGGAUCUUACCACGU	679
4773	AGCUGCUUUAGAAGUAACA	266	4773	AGCUGCUUUAGAAGUAACA	266	4793	UGUUACUUCUAAAGCAGCU	680
4791	AAUGAAGAACGUGGACGUU	267	4791	AAUGAAGAACGUGGACGUU	267	4811	AACGUCCACGUUCUUCAUU	681
4809	UUUUAAUAUAAAGCCUGUU	268	4809	UUUUAAUAUAAAGCCUGUU	268	4829	AACAGGCUUUAUAUUAAAA	682
4827	UUUGUCUUUUGUUGUUGUU	269	4827	UUUGUCUUUUGUUGUUGUU	269	4847	AACAACAACAAAAGACAAA	683
4845	UCAAACGGGAUUCACAGAG	270	4845	UCAAACGGGAUUCACAGAG	270	4865	CUCUGUGAAUCCCGUUUGA	684
4863	GUAUUUGAAAAAUGUAUAU	271	4863	GUAUUUGAAAAAUGUAUAU	271	4883	AUAUACAUUUUUCAAAUAC	685
4881	UAUAUUAAGAGGUCACGGG	272	4881	UAUAUUAAGAGGUCACGGG	272	4901	CCCGUGACCUCUUAAUAUA	686
4899	GGGCUAAUUGCUAGCUGGC	273	4899	GGGCUAAUUGCUAGCUGGC	273	4919	GCCAGCUAGCAAUUAGCCC	687
4917	CUGCCUUUUGCUGUGGGGU	274	4917	CUGCCUUUUGCUGUGGGGU	274	4937	ACCCCACAGCAAAAGGCAG	688
4935	UUUUGUUACCUGGUUUUAA	275	4935	UUUUGUUACCUGGUUUUAA	275	4955	UUAAAACCAGGUAACAAAA	689
4953	AUAACAGUAAAUGUGCCCA	276	4953	AUAACAGUAAAUGUGCCCA	276	4973	UGGGCACAUUUACUGUUAU	690
4971	AGCCUCUUGGCCCCAGAAC	277	4971	AGCCUCUUGGCCCCAGAAC	277	4991	GUUCUGGGGCCAAGAGGCU	691
4989	CUGUACAGUAUUGUGGCUG	278	4989	CUGUACAGUAUUGUGGCUG	278	5009	CAGCCACAAUACUGUACAG	692
5007	GCACUUGCUCUAAGAGUAG	279	5007	GCACUUGCUCUAAGAGUAG	279	5027	CUACUCUUAGAGCAAGUGC	693
5025	GUUGAUGUUGCAUUUUCCU	280	5025	GUUGAUGUUGCAUUUUCCU	280	5045	AGGAAAAUGCAACAUCAAC	694
5043	UUAUUGUUAAAAACAUGUU	281	5043	UUAUUGUUAAAAACAUGUU	281	5063	AACAUGUUUUUAACAAUAA	695

5061	UAGAAGCAAUGAAUGUAUA	282	5061	UAGAAGCAAUGAAUGUAUA	282	5081	UAUACAUUCAUUGCUUCUA	696
5079	AUAAAAGCCUCAACUAGUC	283	5079	AUAAAAGCCUCAACUAGUC	283	5099	GACUAGUUGAGGCUUUUAU	697
5097	CAUUUUUUUCUCCUCUUCU	284	5097	CAUUUUUUUCUCCUCUUCU	284	5117	AGAAGAGGAGAAAAAAAUG	698
5115	UUUUUUUUCAUUAUAUCUA	285	5115	UUUUUUUUCAUUAUAUCUA	285	5135	UAGAUAUAAUGAAAAAAAA	699
5133	AAUUAUUUUGCAGUUGGGC	286	5133	AAUUAUUUUGCAGUUGGGC	286	5153	GCCCAACUGCAAAAUAAUU	700
5151	CAACAGAGAACCAUCCCUA	287	5151	CAACAGAGAACCAUCCCUA	287	5171	UAGGGAUGGUUCUCUGUUG	701
5169	AUUUUGUAUUGAAGAGGGA	288	5169	AUUUUGUAUUGAAGAGGGA	288	5189	UCCCUCUUCAAUACAAAAU	702
5187	AUUCACAUCUGCAUCUUAA	289	5187	AUUCACAUCUGCAUCUUAA	289	5207	UUAAGAUGCAGAUGUGAAU	703
5205	ACUGCUCUUUAUGAAUGAA	290	5205	ACUGCUCUUUAUGAAUGAA	290	5225	UUCAUUCAUAAAGAGCAGU	704
5223	AAAAACAGUCCUCUGUAUG	291	5223	AAAAACAGUCCUCUGUAUG	291	5243	CAUACAGAGGACUGUUUUU	705
5241	GUACUCCUCUUUACACUGG	292	5241	GUACUCCUCUUUACACUGG	292	5261	CCAGUGUAAAGAGGAGUAC	706
5259	GCCAGGGUCAGAGUUAAAU	293	5259	GCCAGGGUCAGAGUUAAAU	293	5279	AUUUAACUCUGACCCUGGC	707
5277	UAGAGUAUAUGCACUUUCC	294	5277	UAGAGUAUAUGCACUUUCC	294	5297	GGAAAGUGCAUAUACUCUA	708
5295	CAAAUUGGGGACAAGGGCU	295	5295	CAAAUUGGGGACAAGGGCU	295	5315	AGCCCUUGUCCCCAAUUUG	709
5313	UCUAAAAAAAAGCCCCAAAA	296	5313	UCUAAAAAAAAGCCCCAAAA	296	5333	UUUUGGGGCUUUUUUUAGA	710
5331	AGGAGAAGAACAUCUGAGA	297	5331	AGGAGAAGAACAUCUGAGA	297	5351	UCUCAGAUGUUCUUCUCCU	711
5349	AACCUCCUCGGCCCUCCCA	298	5349	AACCUCCUCGGCCCUCCCA	298	5369	UGGGAGGGCCGAGGAGGUU	712
5367	AGUCCCUCGCUGCACAAAU	299	5367	AGUCCCUCGCUGCACAAAU	299	5387	AUUUGUGCAGCGAGGGACU	713
5385	UACUCCGCAAGAGAGGCCA	300	5385	UACUCCGCAAGAGAGGCCA	300	5405	UGGCCUCUCUUGCGGAGUA	714
5403	AGAAUGACAGCUGACAGGG	301	5403	AGAAUGACAGCUGACAGGG	301	5423	CCCUGUCAGCUGUCAUUCU	715
5421	GUCUAUGGCCAUCGGGUCG	302	5421	GUCUAUGGCCAUCGGGUCG	302	5441	CGACCCGAUGGCCAUAGAC	716
5439	GUCUCCGAAGAUUUGGCAG	303	5439	GUCUCCGAAGAUUUGGCAG	303	5459	CUGCCAAAUCUUCGGAGAC	717
5457	GGGGCAGAAAACUCUGGCA	304	5457	GGGGCAGAAAACUCUGGCA	304	5477	UGCCAGAGUUUUCUGCCCC	718
5475	AGGCUUAAGAUUUGGAAUA	305	5475	AGGCUUAAGAUUUGGAAUA	305	5495	UAUUCCAAAUCUUAAGCCU	719
5493	AAAGUCACAGAAUCAAGGA	306	5493	AAAGUCACAGAAUCAAGGA	306	5513	UCCUUGAUUCUGUGACUUU	720
5511	AAGCACCUCAAUUUAGUUC	307	5511	AAGCACCUCAAUUUAGUUC	307	5531	GAACUAAAUUGAGGUGCUU	721
5529	CAAACAAGACGCCAACAUU	308	5529	CAAACAAGACGCCAACAUU	308	5549	AAUGUUGGCGUCUUGUUUG	722
5547	UCUCUCCACAGCUCACUUA	309	5547	UCUCUCCACAGCUCACUUA	309	5567	UAAGUGAGCUGUGGAGAGA	723
5565	ACCUCUCUGUGUUCAGAUG	310	5565	ACCUCUCUGUGUUCAGAUG	310	5585	CAUCUGAACACAGAGAGGU	724
5583	GUGGCCUUCCAUUUAUAUG	311	5583	GUGGCCUUCCAUUUAUAUG	311	5603	CAUAUAAAUGGAAGGCCAC	725
5601	GUGAUCUUUGUUUUAUUAG	312	5601	GUGAUCUUUGUUUUAUUAG	312	5621	CUAAUAAAACAAAGAUCAC	726
5619	GUAAAUGCUUAUCAUCUAA	313	5619	GUAAAUGCUUAUCAUCUAA	313	5639	UUAGAUGAUAAGCAUUUAC	727
5637	AAGAUGUAGCUCUGGCCCA	314	5637	AAGAUGUAGCUCUGGCCCA	314	5657	UGGGCCAGAGCUACAUCUU	728
5655	AGUGGGAAAAAUUAGGAAG	315	5655	AGUGGGAAAAAUUAGGAAG	315	5675	CUUCCUAAUUUUUCCCACU	729
5673	GUGAUUAUAAAUCGAGAGG	316	5673	GUGAUUAUAAAUCGAGAGG	316	5693	CCUCUCGAUUUAUAAUCAC	730
5691	GAGUUAUAAUAAUCAAGAU	317	5691	GAGUUAUAAUAAUCAAGAU	317	5711	AUCUUGAUUAUUAUAACUC	731

5709	UUAAAUGUAAAUAAUCAGG	318	5709	UUAAAUGUAAAUAAUCAGG	318	5729	CCUGAUUAUUUACAUUUAA	732
5727	GGCAAUCCCAACACAUGUC	319	5727	GGCAAUCCCAACACAUGUC	319	5747	GACAUGUGUUGGGAUUGCC	733
5745	CUAGCUUUCACCUCCAGGA	320	5745	CUAGCUUUCACCUCCAGGA	320	5765	UCCUGGAGGUGAAAGCUAG	734
5763	AUCUAUUGAGUGAACAGAA	321	5763	AUCUAUUGAGUGAACAGAA	321	5783	UUCUGUUCACUCAAUAGAU	735
5781	AUUGCAAAUAGUCUCUAUU	322	5781	AUUGCAAAUAGUCUCUAUU	322	5801	AAUAGAGACUAUUUGCAAU	736
5799	UUGUAAUUGAACUUAUCCU	323	5799	UUGUAAUUGAACUUAUCCU	323	5819	AGGAUAAGUUCAAUUACAA	737
5817	UAAAACAAAUAGUUUAUAA	324	5817	UAAAACAAAUAGUUUAUAA	324	5837	UUAUAAACUAUUUGUUUUA	738
5835	AAUGUGAACUUAAACUCUA	325	5835	AAUGUGAACUUAAACUCUA	325	5855	UAGAGUUUAAGUUCACAUU	739
5853	AAUUAAUUCCAACUGUACU	326	5853	AAUUAAUUCCAACUGUACU	326	5873	AGUACAGUUGGAAUUAAUU	740
5871	UUUUAAGGCAGUGGCUGUU	327	5871	UUUUAAGGCAGUGGCUGUU	327	5891	AACAGCCACUGCCUUAAAA	741
5889	UUUUAGACUUUCUUAUCAC	328	5889	UUUUAGACUUUCUUAUCAC	328	5909	GUGAUAAGAAAGUCUAAAA	742
5907	CUUAUAGUUAGUAAUGUAC	329	5907	CUUAUAGUUAGUAAUGUAC	329	5927	GUACAUUACUAACUAUAAG	743
5925	CACCUACUCUAUCAGAGAA	330	5925	CACCUACUCUAUCAGAGAA	330	5945	UUCUCUGAUAGAGUAGGUG	744
5943	AAAACAGGAAAGGCUCGAA	331	5943	AAAACAGGAAAGGCUCGAA	331	5963	UUCGAGCCUUUCCUGUUUU	745
5961	AAUACAAGCCAUUCUAAGG	332	5961	AAUACAAGCCAUUCUAAGG	332	5981	CCUUAGAAUGGCUUGUAUU	746
5979	GAAAUUAGGGAGUCAGUUG	333	5979	GAAAUUAGGGAGUCAGUUG	333	5999	CAACUGACUCCCUAAUUUC	747
5997	GAAAUUCUAUUCUGAUCUU	334	5997	GAAAUUCUAUUCUGAUCUU	334	6017	AAGAUCAGAAUAGAAUUUC	748
6015	UAUUCUGUGGUGUCUUUUG	335	6015	UAUUCUGUGGUGUCUUUUG	335	6035	CAAAAGACACCACAGAAUA	749
6033	GCAGCCCAGACAAAUGUGG	336	6033	GCAGCCCAGACAAAUGUGG	336	6053	CCACAUUUGUCUGGGCUGC	750
6051	GUUACACACUUUUUAAGAA	337	6051	GUUACACACUUUUUAAGAA	337	6071	UUCUUAAAAAGUGUGUAAC	751
6069	AAUACAAUUCUACAUUGUC	338	6069	AAUACAAUUCUACAUUGUC	338	6089	GACAAUGUAGAAUUGUAUU	752
6087	CAAGCUUAUGAAGGUUCCA	339	6087	CAAGCUUAUGAAGGUUCCA	339	6107	UGGAACCUUCAUAAGCUUG	753
6105	AAUCAGAUCUUUAUUGUUA	340	6105	AAUCAGAUCUUUAUUGUUA	340	6125	UAACAAUAAAGAUCUGAUU	754
6123	AUUCAAUUUGGAUCUUUCA	341	6123	AUUCAAUUUGGAUCUUUCA	341	6143	UGAAAGAUCCAAAUUGAAU	755
6141	AGGGAUUUUUUUUUUAAAU	342	6141	AGGGAUUUUUUUUUUAAAU	342	6161	AUUUAAAAAAAAAAUCCCU	756
6159	UUAUUAUGGGACAAAGGAC	343	6159	UUAUUAUGGGACAAAGGAC	343	6179	GUCCUUUGUCCCAUAAUAA	757
6177	CAUUUGUUGGAGGGGUGGG	344	6177	CAUUUGUUGGAGGGGUGGG	344	6197	CCCACCCCUCCAACAAAUG	758
6195	GAGGGAGGAACAAUUUUUA	345	6195	GAGGGAGGAACAAUUUUUA	345	6215	UAAAAAUUGUUCCUCCCUC	759
6213	AAAUAUAAAACAUUCCCAA	346	6213	AAAUAUAAAACAUUCCCAA	346	6233	UUGGGAAUGUUUUAUAUUU	760
6231	AGUUUGGAUCAGGGAGUUG	347	6231	AGUUUGGAUCAGGGAGUUG	347	6251	CAACUCCCUGAUCCAAACU	761
6249	GGAAGUUUUCAGAAUAACC	348	6249	GGAAGUUUUCAGAAUAACC	348	6269	GGUUAUUCUGAAAACUUCC	762
6267	CAGAACUAAGGGUAUGAAG	349	6267	CAGAACUAAGGGUAUGAAG	349	6287	CUUCAUACCCUUAGUUCUG	763
6285	GGACCUGUAUUGGGGUCGA	350	6285	GGACCUGUAUUGGGGUCGA	350	6305	UCGACCCCAAUACAGGUCC	764
6303	AUGUGAUGCCUCUGCGAAG	351	6303	AUGUGAUGCCUCUGCGAAG	351	6323	CUUCGCAGAGGCAUCACAU	765
6321	GAACCUUGUGUGACAAAUG	352	6321	GAACCUUGUGUGACAAAUG	352	6341	CAUUUGUCACACAAGGUUC	766
6339	GAGAAACAUUUUGAAGUUU	353	6339	GAGAAACAUUUUGAAGUUU	353	6359	AAACUUCAAAAUGUUUCUC	767

6357	UGUGGUACGACCUUUAGAU	354	6357	UGUGGUACGACCUUUAGAU	354	6377	AUCUAAAGGUCGUACCACA	768
6375	UUCCAGAGACAUCAGCAUG	355	6375	UUCCAGAGACAUCAGCAUG	355	6395	CAUGCUGAUGUCUCUGGAA	769
6393	GGCUCAAAGUGCAGCUCCG	356	6393	GGCUCAAAGUGCAGCUCCG	356	6413	CGGAGCUGCACUUUGAGCC	770
6411	GUUUGGCAGUGCAAUGGUA	357	6411	GUUUGGCAGUGCAAUGGUA	357	6431	UACCAUUGCACUGCCAAAC	771
6429	AUAAAUUUCAAGCUGGAUA	358	6429	AUAAAUUUCAAGCUGGAUA	358	6449	UAUCCAGCUUGAAAUUUAU	772
6447	AUGUCUAAUGGGUAUUUAA	359	6447	AUGUCUAAUGGGUAUUUAA	359	6467	UUAAAUACCCAUUAGACAU	773
6465	AACAAUAAAUGUGCAGUUU	360	6465	AACAAUAAAUGUGCAGUUU	360	6485	AAACUGCACAUUUAUUGUU	774
6483	UUAACUAACAGGAUAUUUA	361	6483	UUAACUAACAGGAUAUUUA	361	6503	UAAAUAUCCUGUUAGUUAA	775
6501	AAUGACAACCUUCUGGUUG	362	6501	AAUGACAACCUUCUGGUUG	362	6521	CAACCAGAAGGUUGUCAUU	776
6519	GGUAGGGACAUCUGUUUCU	363	6519	GGUAGGGACAUCUGUUUCU	363	6539	AGAAACAGAUGUCCCUACC	777
6537	UAAAUGUUUAUUAUGUACA	364	6537	UAAAUGUUUAUUAUGUACA	364	6557	UGUACAUAAUAAACAUUUA	778
6555	AAUACAGAAAAAAAUUUUA	365	6555	AAUACAGAAAAAAAUUUUA	365	6575	UAAAAUUUUUUUCUGUAUU	779
6573	AUAAAAUUAAGCAAUGUGA	366	6573	AUAAAAUUAAGCAAUGUGA	366	6593	UCACAUUGCUUAAUUUUAU	780
6591	AAACUGAAUUGGAGAGUGA	367	6591	AAACUGAAUUGGAGAGUGA	367	6611	UCACUCUCCAAUUCAGUUU	781
6609	AUAAUACAAGUCCUUUAGU	368	6609	AUAAUACAAGUCCUUUAGU	368	6629	ACUAAAGGACUUGUAUUAU	782
6627	UCUUACCCAGUGAAUCAUU	369	6627	UCUUACCCAGUGAAUCAUU	369	6647	AAUGAUUCACUGGGUAAGA	783
6645	UCUGUUCCAUGUCUUUGGA	370	6645	UCUGUUCCAUGUCUUUGGA	370	6665	UCCAAAGACAUGGAACAGA	784
6663	ACAACCAUGACCUUGGACA	371	6663	ACAACCAUGACCUUGGACA	371	6683	UGUCCAAGGUCAUGGUUGU	785
6681	AAUCAUGAAAUAUGCAUCU	372	6681	AAUCAUGAAAUAUGCAUCU	372	6701	AGAUGCAUAUUUCAUGAUU	786
6699	UCACUGGAUGCAAAGAAAA	373	6699	UCACUGGAUGCAAAGAAAA	373	6719	UUUUCUUUGCAUCCAGUGA	787
6717	AUCAGAUGGAGCAUGAAUG	374	6717	AUCAGAUGGAGCAUGAAUG	374	6737	CAUUCAUGCUCCAUCUGAU	788
6735	GGUACUGUACCGGUUCAUC	375	6735	GGUACUGUACCGGUUCAUC	375	6755	GAUGAACCGGUACAGUACC	789
6753	CUGGACUGCCCCAGAAAAA	376	6753	CUGGACUGCCCCAGAAAAA	376	6773	UUUUUCUGGGGCAGUCCAG	790
6771	AUAACUUCAAGCAAACAUC	377	6771	AUAACUUCAAGCAAACAUC	377	6791	GAUGUUUGCUUGAAGUUAU	791
6789	CCUAUCAACAACAAGGUUG	378	6789	CCUAUCAACAACAAGGUUG	378	6809	CAACCUUGUUGUUGAUAGG	792
6807	GUUCUGCAUACCAAGCUGA	379	6807	GUUCUGCAUACCAAGCUGA	379	6827	UCAGCUUGGUAUGCAGAAC	793
6825	AGCACAGAAGAUGGGAACA	380	6825	AGCACAGAAGAUGGGAACA	380	6845	UGUUCCCAUCUUCUGUGCU	794
6843	ACUGGUGGAGGAUGGAAAG	381	6843	ACUGGUGGAGGAUGGAAAG	381	6863	CUUUCCAUCCUCCACCAGU	795
6861	GGCUCGCUCAAUCAAGAAA	382	6861	GGCUCGCUCAAUCAAGAAA	382	6881	UUUCUUGAUUGAGCGAGCC	796
6879	AAUUCUGAGACUAUUAAUA	383	6879	AAUUCUGAGACUAUUAAUA	383	6899	UAUUAAUAGUCUCAGAAUU	797
6897	AAAUAAGACUGUAGUGUAG	384	6897	AAAUAAGACUGUAGUGUAG	384	6917	CUACACUACAGUCUUAUUU	798
6915	GAUACUGAGUAAAUCCAUG	385	6915	GAUACUGAGUAAAUCCAUG	385	6935	CAUGGAUUUACUCAGUAUC	799
6933	GCACCUAAACCUUUUGGAA	386	6933	GCACCUAAACCUUUUGGAA	386	6953	UUCCAAAAGGUUUAGGUGC	800
6951	AAAUCUGCCGUGGGCCCUC	387	6951	AAAUCUGCCGUGGGCCCUC	387	6971	GAGGGCCCACGGCAGAUUU	801
6969	CCAGAUAGCUCAUUUCAUU	388	6969	CCAGAUAGCUCAUUUCAUU	388	6989	AAUGAAAUGAGCUAUCUGG	802
6987	UAAGUUUUUCCCUCCAAGG	389	6987	UAAGUUUUUCCCUCCAAGG	389	7007	CCUUGGAGGGAAAAACUUA	803

7005	GUAGAAUUUGCAAGAGUGA	390	7005	GUAGAAUUUGCAAGAGUGA	390	7025	UCACUCUUGCAAAUUCUAC	804
7023	ACAGUGGAUUGCAUUUCUU	391	7023	ACAGUGGAUUGCAUUUCUU	391	7043	AAGAAAUGCAAUCCACUGU	805
7041	UUUGGGGAAGCUUUCUUUU	392	7041	UUUGGGGAAGCUUUCUUUU	392	7061	AAAAGAAAGCUUCCCCAAA	806
7059	UGGUGGUUUUGUUUAUUAU	393	7059	UGGUGGUUUUGUUUAUUAU	393	7079	AUAAUAAACAAAACCACCA	807
7077	UACCUUCUUAAGUUUUCAA	394	7077	UACCUUCUUAAGUUUUCAA	394	7097	UUGAAAACUUAAGAAGGUA	808
7095	ACCAAGGUUUGCUUUUGUU	395	7095	ACCAAGGUUUGCUUUUGUU	395	7115	AACAAAAGCAAACCUUGGU	809
7113	UUUGAGUUACUGGGGUUAU	396	7113	UUUGAGUUACUGGGGUUAU	396	7133	AUAACCCCAGUAACUCAAA	810
7131	UUUUUGUUUUAAAUAAAAA	397	7131	UUUUUGUUUUAAAUAAAAA	397	7151	UUUUUAUUUAAAACAAAAA	811
7149	AUAAGUGUACAAUAAGUGU	398	7149	AUAAGUGUACAAUAAGUGU	398	7169	ACACUUAUUGUACACUUAU	812
7167	UUUUUGUAUUGAAAGCUUU	399	7167	UUUUUGUAUUGAAAGCUUU	399	7187	AAAGCUUUCAAUACAAAAA	813
7185	UUGUUAUCAAGAUUUUCAU	400	7185	UUGUUAUCAAGAUUUUCAU	400	7205	AUGAAAAUCUUGAUAACAA	814
7203	UACUUUUACCUUCCAUGGC	401	7203	UACUUUUACCUUCCAUGGC	401	7223	GCCAUGGAAGGUAAAAGUA	815
7221	CUCUUUUUAAGAUUGAUAC	402	7221	CUCUUUUUAAGAUUGAUAC	402	7241	GUAUCAAUCUUAAAAAGAG	816
7239	CUUUUAAGAGGUGGCUGAU	403	7239	CUUUUAAGAGGUGGCUGAU	403	7259	AUCAGCCACCUCUUAAAAG	817
7257	UAUUCUGCAACACUGUACA	404	7257	UAUUCUGCAACACUGUACA	404	7277	UGUACAGUGUUGCAGAAUA	818
7275	ACAUAAAAAAUACGGUAAG	405	7275	ACAUAAAAAAUACGGUAAG	405	7295	CUUACCGUAUUUUUUAUGU	819
7293	GGAUACUUUACAUGGUUAA	406	7293	GGAUACUUUACAUGGUUAA	406	7313	UUAACCAUGUAAAGUAUCC	820
7311	AGGUAAAGUAAGUCUCCAG	407	7311	AGGUAAAGUAAGUCUCCAG	407	7331	CUGGAGACUUACUUUACCU	821
7329	GUUGGCCACCAUUAGCUAU	408	7329	GUUGGCCACCAUUAGCUAU	408	7349	AUAGCUAAUGGUGGCCAAC	822
7347	UAAUGGCACUUUGUUUGUG	409	7347	UAAUGGCACUUUGUUUGUG	409	7367	CACAAACAAAGUGCCAUUA	823
7365	GUUGUUGGAAAAAGUCACA	410	7365	GUUGUUGGAAAAAGUCACA	410	7385	UGUGACUUUUUCCAACAAC	824
7383	AUUGCCAUUAAACUUUCCU	411	7383	AUUGCCAUUAAACUUUCCU	411	7403	AGGAAAGUUUAAUGGCAAU	825
7401	UUGUCUGUCUAGUUAAUAU	412	7401	UUGUCUGUCUAGUUAAUAU	412	7421	AUAUUAACUAGACAGACAA	826
7419	UUGUGAAGAAAAAUAAAGU	413	7419	UUGUGAAGAAAAAUAAAGU	413	7439	ACUUUAUUUUUCUUCACAA	827
7433	AAAGUACAGUGUGAGAUAC	414	7433	AAAGUACAGUGUGAGAUAC	414	7453	GUAUCUCACACUGUACUUU	828

The 3'-ends of the Upper sequence and the Lower sequence of the siNA construct can include an overhang sequence, for example about 1, 2, 3, or 4 nucleotides in length, preferably 2 nucleotides in length, wherein the overhanging sequence of the lower sequence is optionally complementary to a portion of the target sequence. The upper sequence is also referred to as the sense strand, whereas the lower sequence is also referred to as the antisense strand. The upper and lower sequences in the Table can further comprise a chemical modification having Formulae I-VII or any combination thereof.

Table III: BCL2 Synthetic Modified siNA constructs

Target Pos	Target	Seq ID	RPI #	Aliases	Sequence	Seq ID
2098	UGGCUGUCUCUGAAGACUCUGCU	829	30997	BCL2:2100U21 siRNA sense	GCUGUCUCUGAAGACUCUGTT	833
3220	CAGGGAUGAUCAACAGGGUAGUG	830	30998	BCL2:3222U21 siRNA sense	GGGAUGAUCAACAGGGUAGTT	834
4426	CUUUACGUGGCCUGUUUCAACAC	831	30999	BCL2:4428U21 siRNA sense	UUACGUGGCCUGUUUCAACTT	835
6231	AGUUUGGAUCAGGGAGUUGGAAG	832	31000	BCL2:6233U21 siRNA sense	UUUGGAUCAGGGAGUUGGATT	836
2098	UGGCUGUCUCUGAAGACUCUGCU	829	31073	BCL2:2118L21 siRNA (2100C) antisense	CAGAGUCUUCAGAGACAGCTT	837
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31074	BCL2:3240L21 siRNA (3222C) antisense	CUACCCUGUUGAUCAUCCCTT	838
4426	CUUUACGUGGCCUGUUUCAACAC	831	31075	BCL2:4446L21 siRNA (4428C) antisense	GUUGAAACAGGCCACGUAATT	839
6231	AGUUUGGAUCAGGGAGUUGGAAG	832	31076	BCL2:6251L21 siRNA (6233C) antisense	UCCAACUCCCUGAUCCAAATT	840
2098	UGGCUGUCUCUGAAGACUCUGCU	829	30737	BCL2:2100U21 siRNA stab04 sense	B GcuGucucuGAAGAcucuGTT B	841
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31368	BCL2:3222U21 siRNA stab04 sense	B GGGAuGAucAAcAGGGuAGTT B	842
4426	CUUUACGUGGCCUGUUUCAACAC	831	30739	BCL2:4428U21 siRNA stab04 sense	B uuAcGuGGccuGuuucAAcTT B	843
6231	AGUUUGGAUCAGGGAGUUGGAAG	832	30740	BCL2:6233U21 siRNA stab04 sense	B uuuGGAucAGGGAGuuGGATT B	844
2098	UGGCUGUCUCUGAAGACUCUGCU	829	30741	BCL2:2118L21 siRNA (2100C) stab05 antisense	cAGAGucuucAGAGAcAGcTsT	845
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31369	BCL2:3240L21 siRNA (3222C) stab05 antisense	cuAcccuGuuGAucAucccTsT	846
4426	CUUUACGUGGCCUGUUUCAACAC	831	30743	BCL2:4446L21 siRNA (4428C) stab05 antisense	GuuGAAAcAGGccAcGuAATsT	847
6231	AGUUUGGAUCAGGGAGUUGGAAG	832	30744	BCL2:6251L21 siRNA (6233C) stab05 antisense	uccAAcucccuGAuccAAATsT	848
2098	UGGCUGUCUCUGAAGACUCUGCU	829		BCL2:2100U21 siRNA stab07 sense	B GcuGucucuGAAGAcucuGTT B	849
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31372	BCL2:3222U21 siRNA stab07 sense	B GGGAuGAucAAcAGGGuAGTT B	850
4426	CUUUACGUGGCCUGUUUCAACAC	831		BCL2:4428U21 siRNA stab07 sense	B uuAcGuGGccuGuuucAAcTT B	851
6231	AGUUUGGAUCAGGGAGUUGGAAG	832		BCL2:6233U21 siRNA stab07 sense	B uuuGGAucAGGGAGuuGGATT B	852
2098	UGGCUGUCUCUGAAGACUCUGCU	829		BCL2:2118L21 siRNA (2100C) stab11 antisense	cAGAGucuucAGAGAcAGcTsT	853
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31373	BCL2:3240L21 siRNA (3222C) stab11 antisense	cuAcccuGuuGAucAucccTsT	854
4426	CUUUACGUGGCCUGUUUCAACAC	831		BCL2:4446L21 siRNA (4428C) stab11 antisense	GuuGAAAcAGGccAcGuAATsT	855
6231	AGUUUGGAUCAGGGAGUUGGAAG	832		BCL2:6251L21 siRNA (6233C) stab11 antisense	uccAAcucccuGAuccAAATsT	856
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31370	BCL2:3222U21 siRNA inv stab04	B GAuGGGAcAAcuAGuAGGGTT B	857
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31371	BCL2:3240L21 siRNA (3222C) inv stab05	cccuAcuAGuuGucccAucTsT	858
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31374	BCL2:3222U21 siRNA inv stab07	B GAuGGGAcAAcuAGuAGGGTT B	859
3220	CAGGGAUGAUCAACAGGGUAGUG	830	31375	BCL2:3240L21 siRNA (3222C) inv stab11	cccuAcuAGuuGucccAucTsT	860

Uppercase = ribonucleotide
u,c = 2'-deoxy-2'-fluoro U,C
T = thymidine
B = inverted deoxy abasic
s = phosphorothioate linkage
A = deoxy Adenosine
G = deoxy Guanosine

Table IV

Non-limiting examples of Stabilization Chemistries for chemically modified siNA constructs

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 1"	Ribo	Ribo	-	5 at 5'-end 1 at 3'-end	S/AS
"Stab 2"	Ribo	Ribo	-	All linkages	Usually AS
"Stab 3"	2'-fluoro	Ribo	-	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4"	2'-fluoro	Ribo	5' and 3'-ends	-	Usually S
"Stab 5"	2'-fluoro	Ribo	-	1 at 3'-end	Usually AS
"Stab 6"	2'-O-Methyl	Ribo	5' and 3'-ends	-	Usually S
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	-	Usually S
"Stab 8"	2'-fluoro	2'-O-Methyl	-	1 at 3'-end	Usually AS
"Stab 9"	Ribo	Ribo	5' and 3'-ends	-	Usually S
"Stab 10"	Ribo	Ribo	-	1 at 3'-end	Usually AS
"Stab 11"	2'-fluoro	2'-deoxy	-	1 at 3'-end	Usually AS

CAP = any terminal cap, see for example **Figure 10**.

All Stab 1-11 chemistries can comprise 3'-terminal thymidine (TT) residues

All Stab 1-11 chemistries typically comprise 21 nucleotides, but can vary as described herein.

S = sense strand

AS = antisense strand

(400/086)

Table V

A. 2.5 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 µL	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 µL	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 µL	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 µL	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 µL	45 sec	233 min	465 sec
Acetic Anhydride	655	124 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 µL	5 sec	5 sec	5 sec
TCA	700	732 µL	10 sec	10 sec	10 sec
Iodine	20.6	244 µL	15 sec	15 sec	15 sec
Beaucage	7.7	232 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 µmol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 µL	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 µL	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 µL	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 µL	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 µL	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 µL	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 µL	NA	NA	NA

- Wait time does not include contact time during delivery.
- Tandem synthesis utilizes double coupling of linker molecule

(400/086)

CLAIMS

What we claim is:

1. A short interfering nucleic acid (siNA) molecule that down-regulates expression of a BCL2 gene by RNA interference.

2. The siNA molecule of claim 1, wherein said BCL2 gene encodes sequence comprising Genbank Accession number NM_000633.

3. The siNA molecule of claim 1, wherein said siNA molecule comprises no ribonucleotides.

4. The siNA molecule of claim 1, wherein said siNA molecule comprises ribonucleotides.

5. The siNA molecule of claim 1, wherein said siNA molecule is double stranded.

6. The siNA molecule of claim 5, wherein said siNA molecule comprises an antisense strand comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a BCL2 protein, and wherein said siNA molecule further comprises a sense strand, wherein said sense strand comprises a nucleotide sequence of a BCL2 gene or a portion thereof.

7. The siNA molecule of claim 6, wherein said antisense strand and said sense strand each comprise about 19 to about 29 nucleotides, and wherein said antisense strand and said sense strand share at least about 19 complementary nucleotides.

8. The siNA molecule of claim 5, wherein said siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a BCL2 protein, and wherein said siNA further comprises a sense region, wherein said sense region comprises a nucleotide sequence of a BCL2 gene or a portion thereof.

9. The siNA molecule of claim 8, wherein said antisense region and said sense region each comprise about 19 to about 29 nucleotides, and wherein said antisense region and said sense region share at least about 19 complementary nucleotides.

10. The siNA molecule of claim 1, wherein said siNA molecule is single stranded.

(400/086)

11. The siNA molecule of claim 10, wherein said siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a BCL2 protein.

12. The siNA molecule of claim 11, wherein said siNA molecule comprises a sequence having about 19 to about 29 nucleotides.

13. The siNA molecule of claim 1, wherein said siNA molecule comprises a sense region and an antisense region and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof encoding a BCL2 protein and said sense region comprises a nucleotide sequence complementary to said antisense region.

14. The siNA molecule of claim 1, wherein said siNA molecule is assembled from two oligonucleotide fragments wherein one fragment comprises a sense region and a second fragment comprises an antisense region of said siNA molecule.

15. The siNA molecule of claim 13, wherein said sense region and said antisense region comprise separate oligonucleotides.

16. The siNA molecule of claim 13, wherein said sense region and said antisense region are connected via a linker molecule.

17. The siNA molecule of claim 16, wherein said linker molecule is a polynucleotide linker.

18. The siNA molecule of claim 16, wherein said linker molecule is a non-nucleotide linker.

19. The siNA molecule of claim 13, wherein said sense region comprises a 3'-terminal overhang and said antisense region comprises a 3'-terminal overhang.

20. The siNA molecule of claim 19, wherein said 3'-terminal overhangs each comprise about 2 nucleotides.

21. The siNA molecule of claim 19, wherein the 3'-terminal overhang of the antisense region is complementary to RNA encoding a BCL2 protein.

22. The siNA molecule of claim 13, wherein said sense region comprises one or more 2'-O-methyl pyrimidine nucleotides and one or more 2'-deoxy purine nucleotides.

23. The siNA molecule of claim 13, wherein any pyrimidine nucleotides present in said sense region comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein any purine nucleotides present in said sense region comprise 2'-deoxy purine nucleotides.

24. The siNA molecule of claim 19, wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

25. The siNA molecule of claim 13, wherein said sense region comprises a 3'-end and a 5'-end, and wherein a terminal cap moiety is present at the 5'-end, the 3'-end, or both of the 5' and 3' ends of said sense region.

26. The siNA molecule of claim 25, wherein said terminal cap moiety is an inverted deoxy abasic moiety.

27. The siNA molecule of claim 13, wherein said antisense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides and one or more 2'-O-methyl purine nucleotides.

28. The siNA molecule of claim 13, wherein any pyrimidine nucleotides present in said antisense region comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein any purine nucleotides present in said antisense region comprise 2'-O-methyl purine nucleotides.

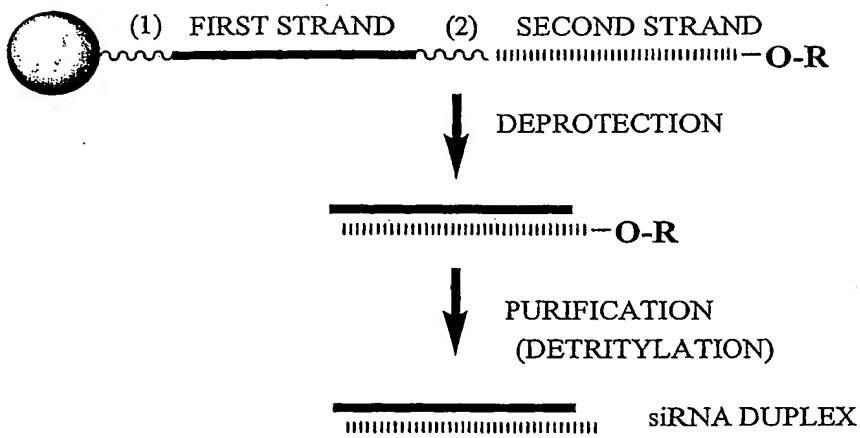
29. The siNA molecule of claim 19, wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

30. The siNA molecule of claim 28, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region.

31. The siNA molecule of claim 13, wherein said antisense region comprises a glyceryl modification at the 3' end of said antisense region.

32. The siNA molecule of claim 19, wherein said 3'-terminal overhangs comprise deoxyribonucleotides.

Figure 1

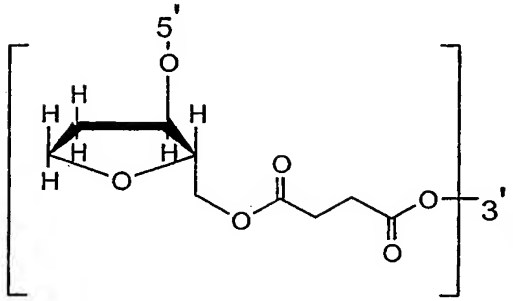


= SOLID SUPPORT

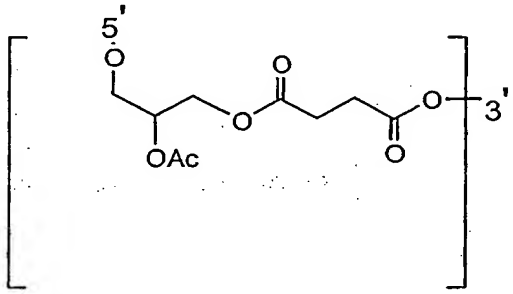
R = TERMINAL PROTECTING GROUP
FOR EXAMPLE:
DIMETHOXYTRITYL (DMT)

(1) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)

(2) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)



INVERTED DEOXYABASIC SUCCINATE LINKAGE



GLYCERYL SUCCINATE LINKAGE

Figure 2

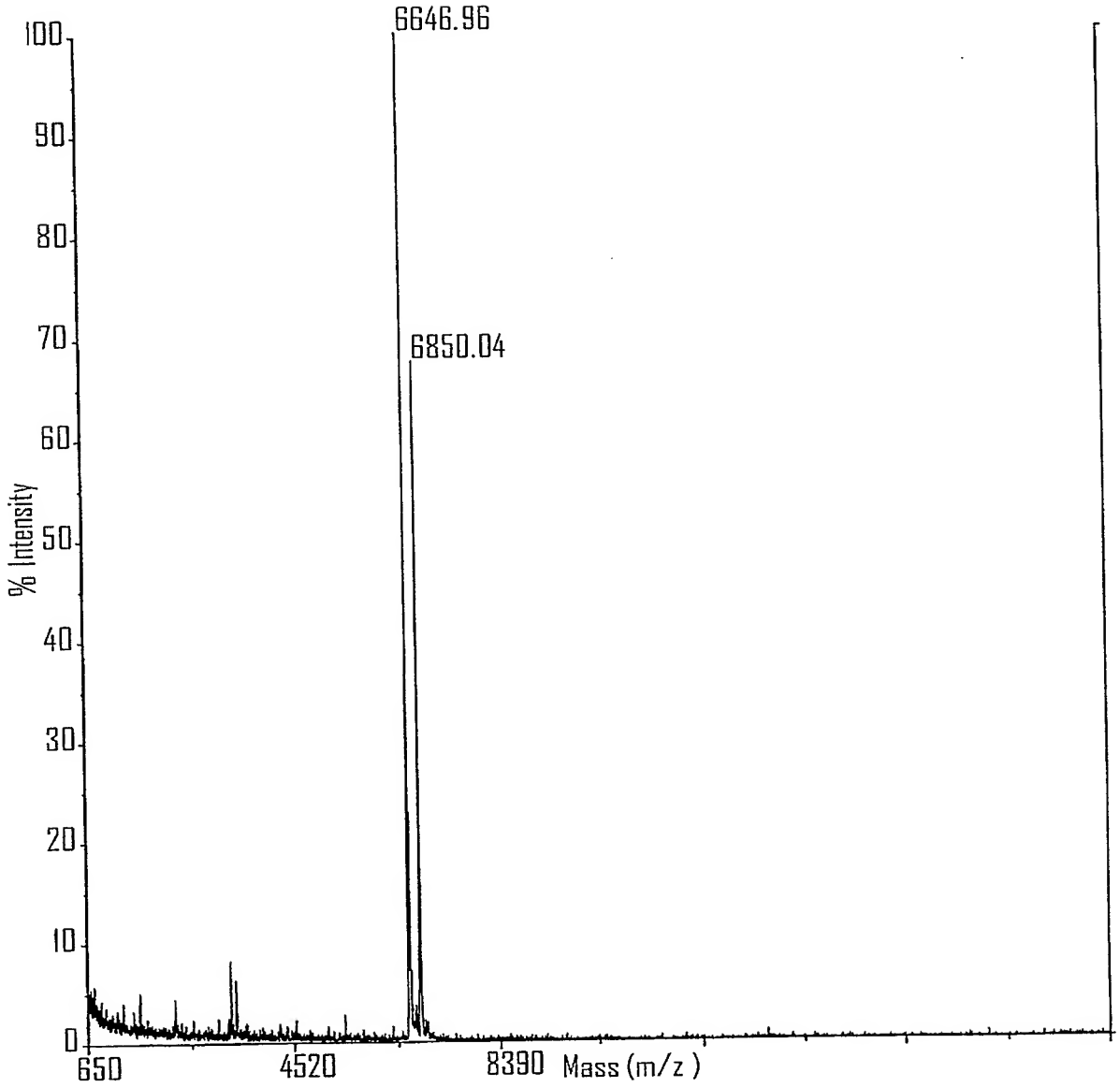

6646.96
6850.04
100
90
80
70
60
50
40
30
20
10
0
% Intensity
650
4520
8390
Mass (m/z)

Figure 3

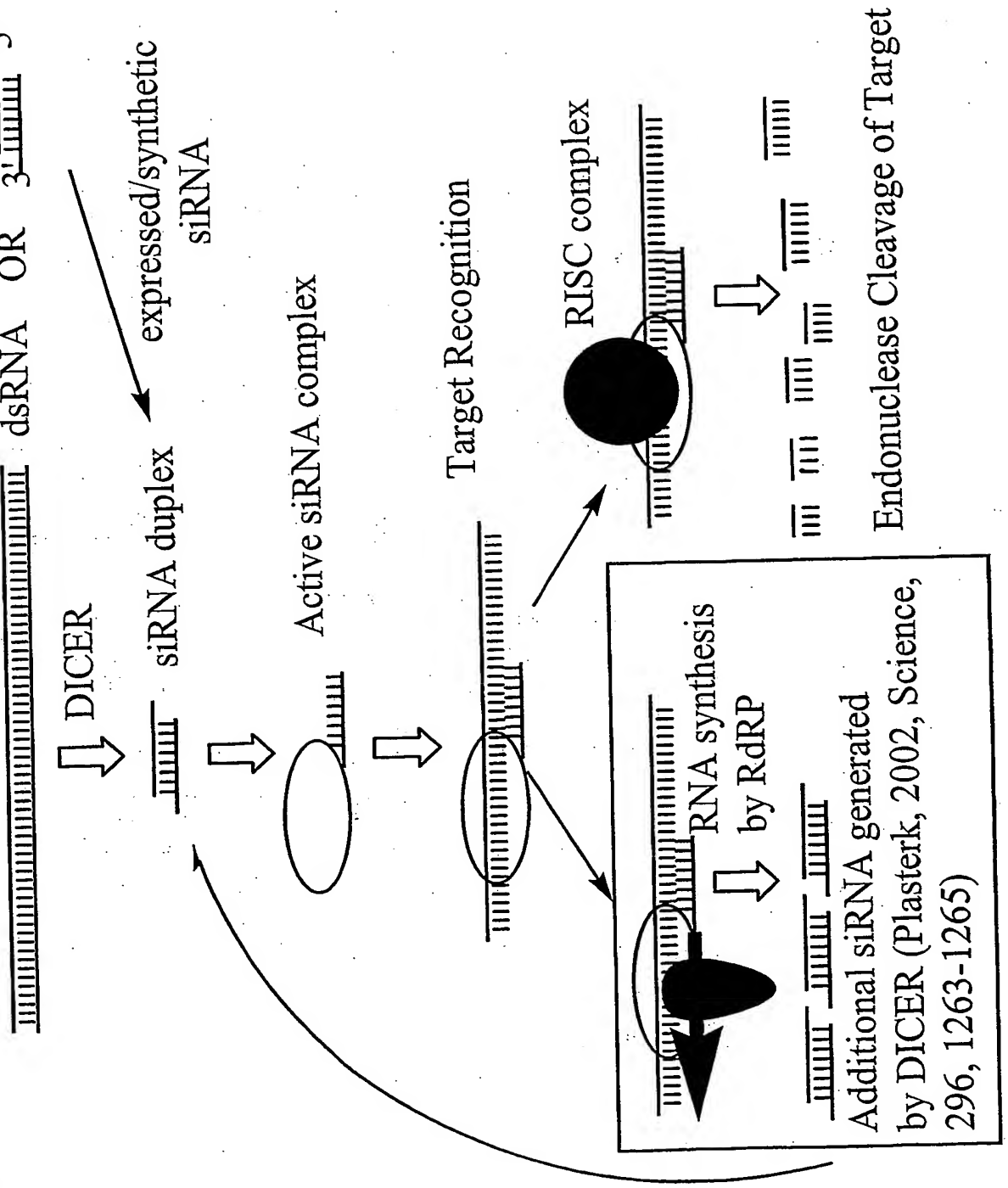


Figure 4

A

SENSE STRAND (SEQ ID NO 861)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- $N_S N_S N_S N_S N N N N N N N N N N N N N N N_S N_S N_S (N_S N)$ -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N $N_S N_S N_S N_S N$ -5'

ANTISENSE STRAND (SEQ ID NO 862)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

B

SENSE STRAND (SEQ ID NO 863)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- N N N N N N N N N N N N N N N N N N N N (N N) -3'

3'- L-(N N) N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 864)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

C

SENSE STRAND (SEQ ID NO 865)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 866)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

D

SENSE STRAND (SEQ ID NO 867)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 868)
ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (N N)

E

SENSE STRAND (SEQ ID NO 869)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-(N N) N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 870)
ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (N N)

F

SENSE STRAND (SEQ ID NO 867)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 871)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

POSITIONS (NN) CAN COMPRISE ANY NUCLEOTIDE, SUCH AS DEOXYNUCLEOTIDES (eg. THYMIDINE) OR UNIVERSAL BASES
B = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP THAT IS OPTIONALLY PRESENT
L = GLYCERYL MOIETY THAT IS OPTIONALLY PRESENT
S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE

Figure 5

A

SENSE STRAND (SEQ ID NO 872)

5'- A_S u_S c_S A_S u u u A u u u u u u u A c $A_S u_S u_S T_S T$ -3'

3'- L-$T_S T$ u A G u A A A u A A AA A A_S u_S G_S u_S A A -5'

ANTISENSE STRAND (SEQ ID NO 873)

B

SENSE STRAND (SEQ ID NO 874)

5'- A u c A u u u A u u u u u u u A c A u u *TT* -3'

3'- L-*TT* u A G u A A A u A A AA A A u G u A A -5'

ANTISENSE STRAND (SEQ ID NO 875)

C

SENSE STRAND (SEQ ID NO 876)

5'- iB-A u c A u u u A u u u u u u u A c A u u *TT*-iB -3'

3'- L-T_S *T u* A G u A A A u A A AA A A u G u A A -5'

ANTISENSE STRAND (SEQ ID NO 877)

D

SENSE STRAND (SEQ ID NO 878)

5'- iB-*A u c A u u u A u u u u u u u A c A u u TT*-iB -3'

3'- L-T_S *T u* a g u a a a u a a a a a a u a u a a -5'

ANTISENSE STRAND (SEQ ID NO 879)

E

SENSE STRAND (SEQ ID NO 880)

5'- iB-A *u c* A *u u u* A *u u u u u u u* A *c* A *u u TT*-iB -3'

3'- L-*TT u* a g u a a a u a a a a a a u a u a a -5'

ANTISENSE STRAND (SEQ ID NO 881)

F

SENSE STRAND (SEQ ID NO 878)

5'- iB-*A u c A u u u A u u u u u u u A c A u u TT*-iB -3'

3'- L-T_S *T u A G u A A A u A A AA A A u G u A A* -5'

ANTISENSE STRAND (SEQ ID NO 882)

lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro
italic lower case = 2'-deoxy-2'-fluoro
underline = 2'-O-methyl

ITALIC UPPER CASE = DEOXY
B = INVERTED DEOXYABASIC
L = GLYCERYL MOIETY OPTIONALLY PRESENT
S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE

Figure 6

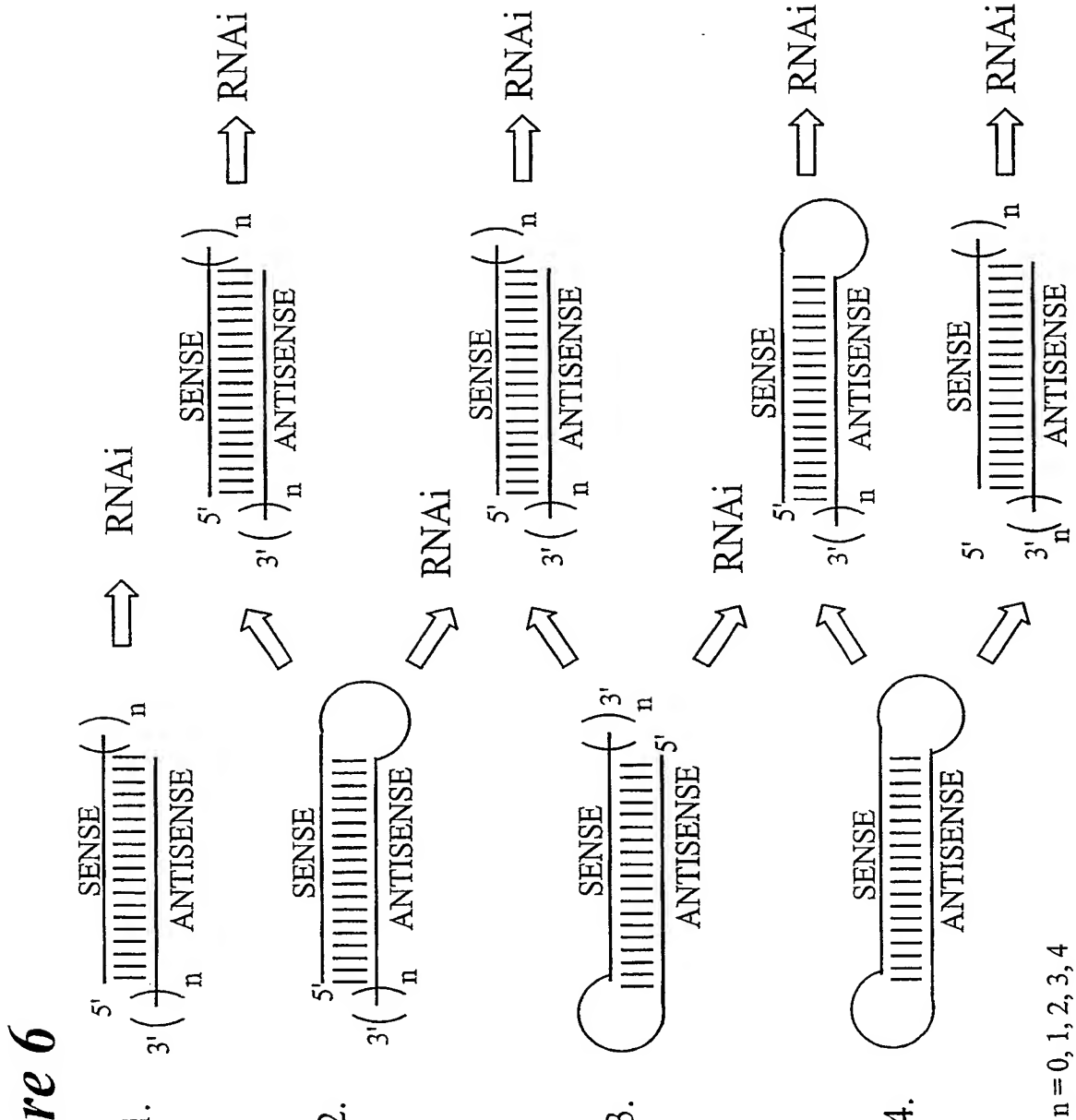


Figure 7

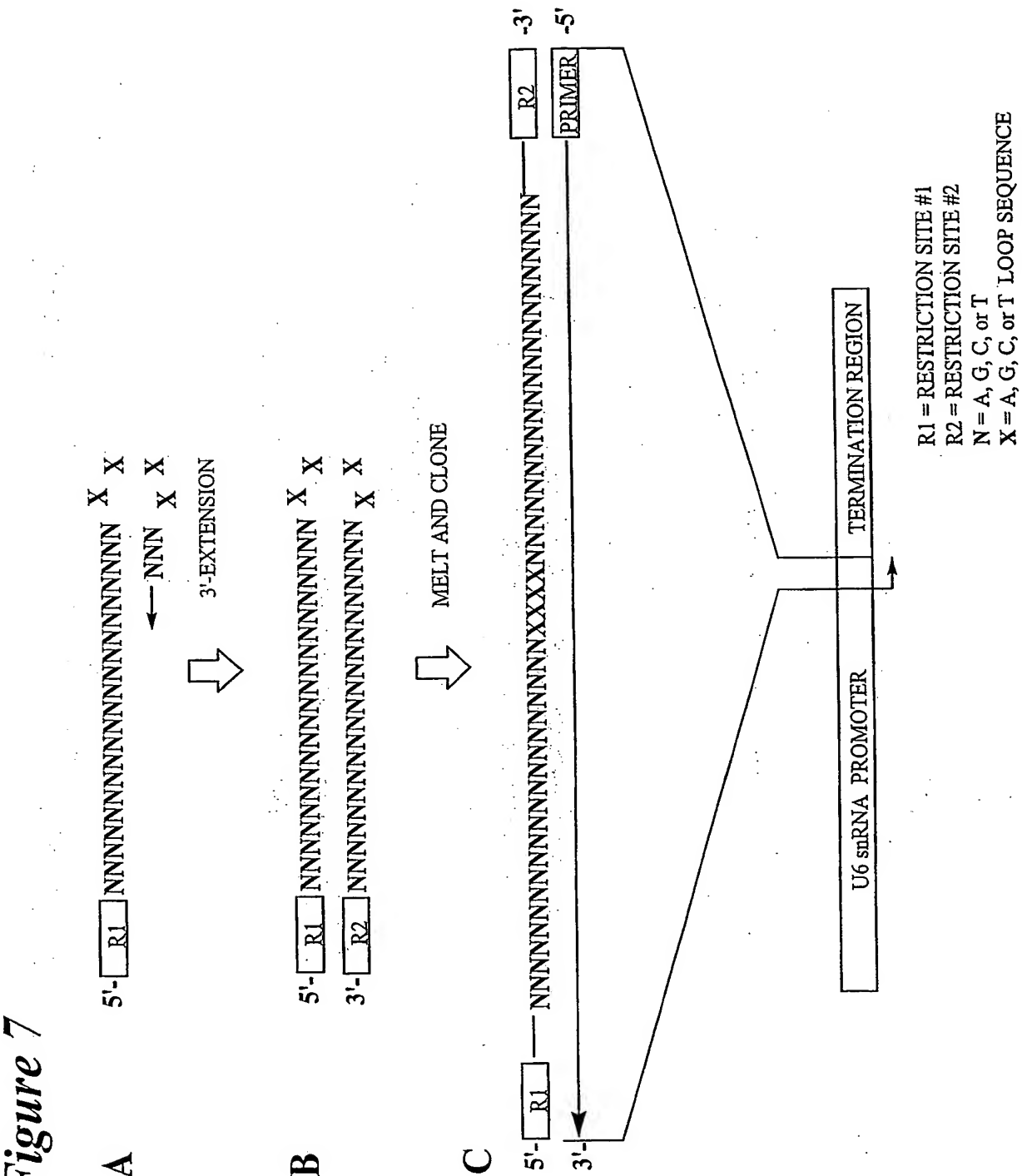


Figure 8

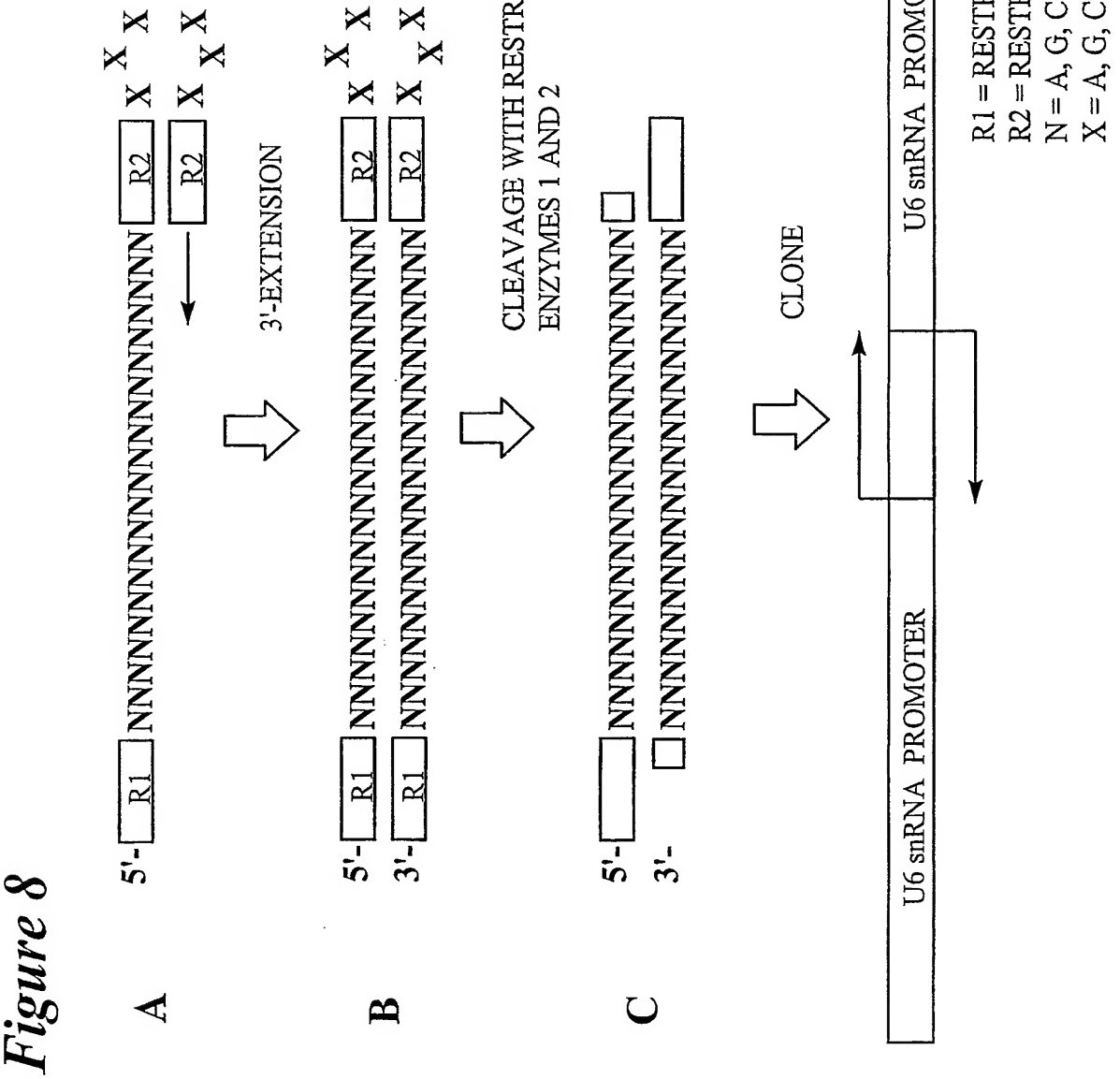


Figure 9: Target site Selection using siRNA

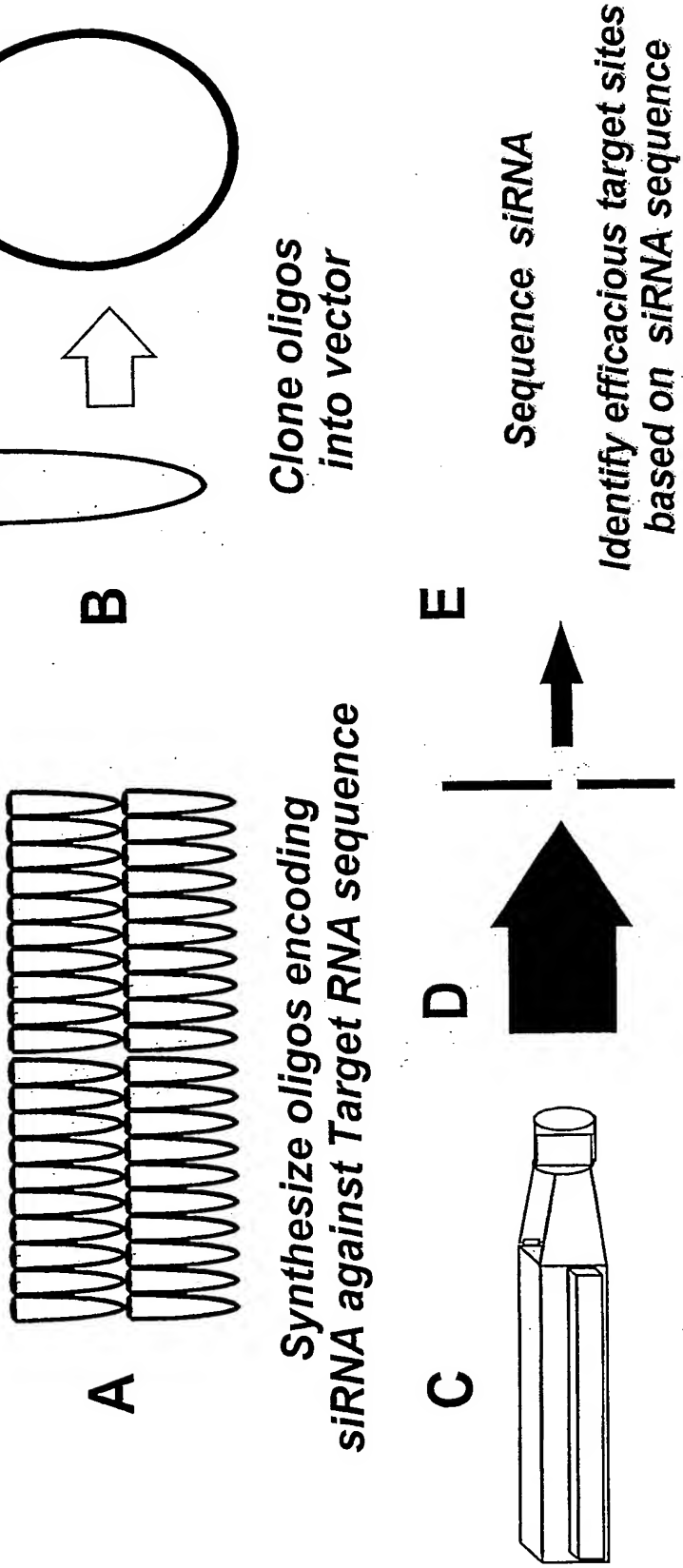
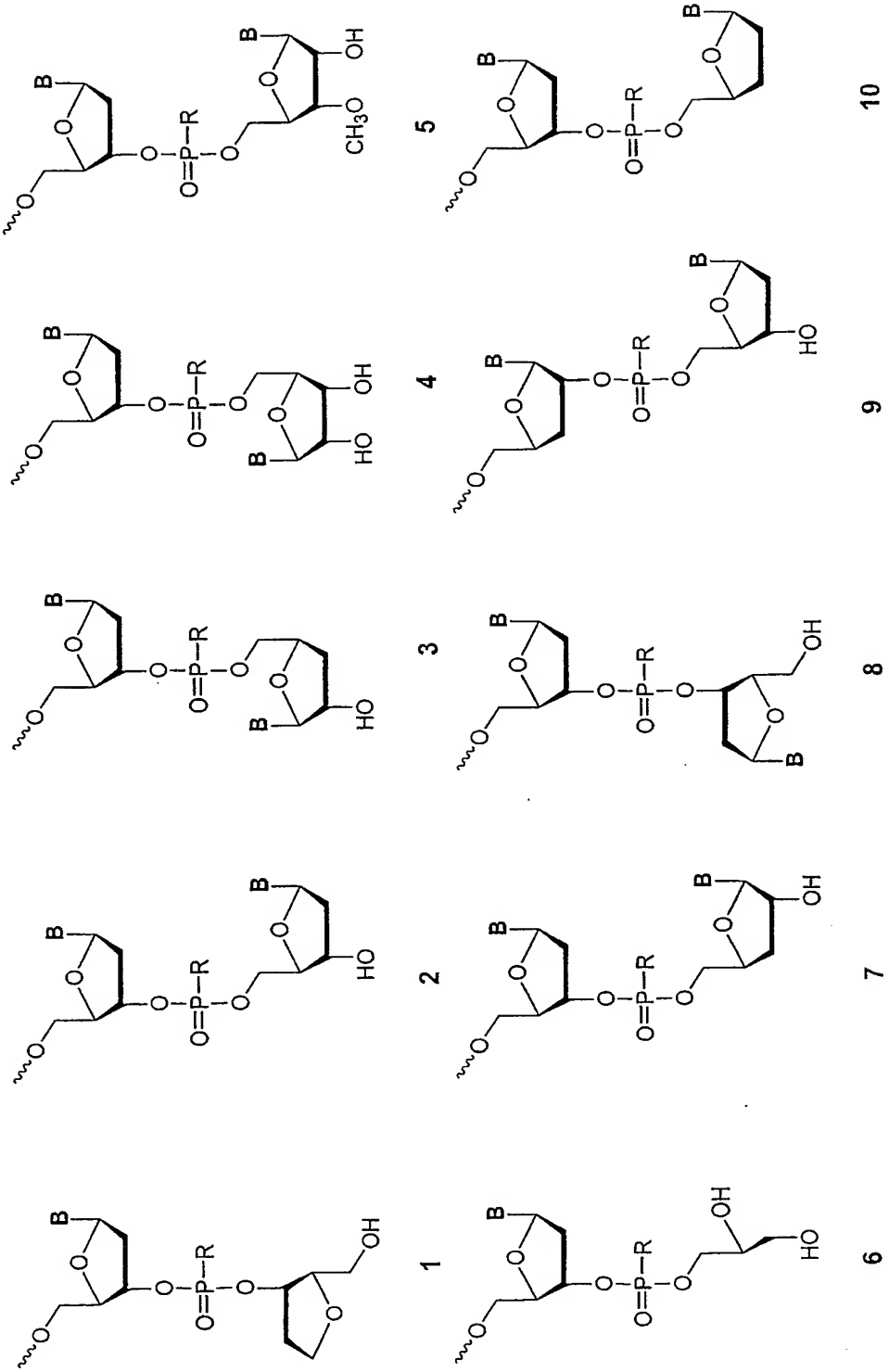
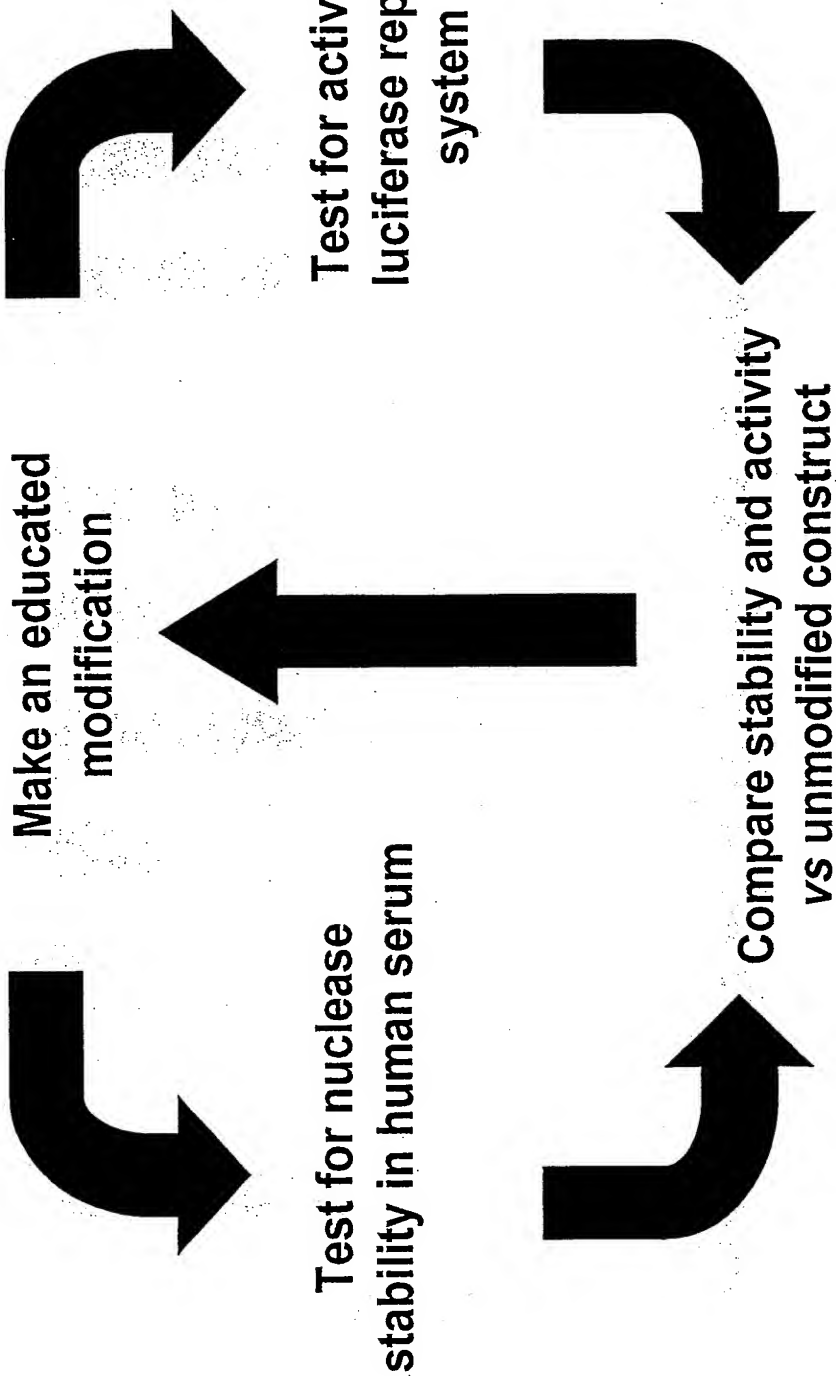


Figure 10



R = O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl
B = Independently any nucleotide base, either naturally occurring or chemically modified, or optionally H (abasic).

Figure 11: Modification Strategy



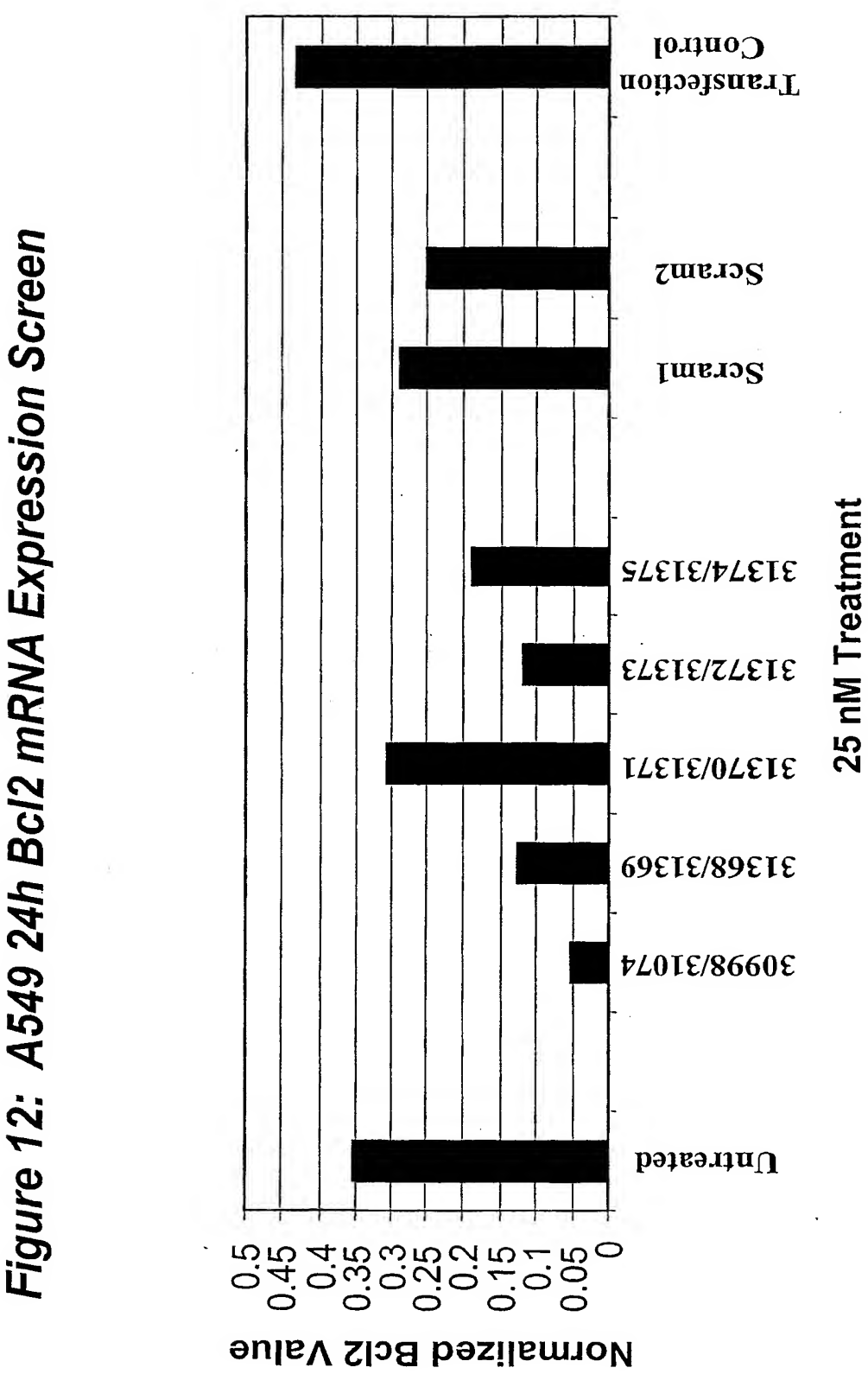


Figure 12: A549 24h Bcl2 mRNA Expression Screen

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60/396,905	18 July 2002 (18.07.2002)	US
60/406,784	29 August 2002 (29.08.2002)	US
60/408,378	5 September 2002 (05.09.2002)	US
60/409,293	9 September 2002 (09.09.2002)	US
60/440,129	15 January 2003 (15.01.2003)	US

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(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF BCL2 GENE EXPRESSION USING SHORT INTERFERING NUCLEIC ACID (siNA)

(57) Abstract: The present invention concerns methods and reagents useful in modulating BCL2 gene expression in a variety of applications, including use in therapeutic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), doublestranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against BCL2, BCL-XL, MCL-1, BCL2-Ll, CED-9, BAG-1, ElB-194 and/or BCL-A1 gene expression, useful in the treatment of cancer and any other condition that responds to modulation of BCL2, BCL-XL, MCL-1, BCL2-Ll, CED-9, BAG-1, E1B-194 and/or BCL-A1 expression.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/04908

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C12P 19/34; C12N 15/63; C07H 21/02, 21/04; A01N 43/04

US CL : 435/6, 91.1, 91.31, 455; 514/44; 536/23.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.31, 455; 514/44; 536/23.1, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

West, Dialog

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 6,414,134 B1 (REED, J) 02 July 2002 (02.07.2002), see entire document, especially figures 2, 7, 8, 13; co. 6-9; claims 1-3.	1-32
Y	FIRE, A. et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans, Nature, 19 February 1998, Vol. 391, pages 806-811, see especially table 1 on page 807; figure 1 on page 808.	1-32
Y	NYKANEN, A. et al. ATP requirements and small interfering RNA strucutre in the RNA interference pathway, Cell, 2 November 2001, Vol. 107, pages 309-321, entire document, especially figure 6 on page 316.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/04908

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim
Y	US 5,877,309 A (MCKAY et al) 02 March 1999 (02.03.1999), see especially col. 6-15.	1-32

Form PCT/ISA/210 (continuation of second sheet) (July 1998)★